THE DETERMINATION OF ACETYLCHOLINESTERASE ACTIVITY AND ITS APPLICATION IN THE PRESENCE OF SOME BENZOTRIAZINIUM COMPOUNDS.

A THESIS

submitted by

GORDON C. JEFFERSON.

for the degree of

DOCTOR OF PHILOSOPHY

in the

HERIOT-WATT UNIVERSITY.

Department of Pharmacy

Heriot-Watt University

Edinburgh.

September, 1974.

ABSTRACT

The Introduction to this thesis discussed factors which were considered in the selection of the Warburg manometric and pH - stat methods for possible use in the determination of the anti-acetyl-cholinesterase activities of some benzotriazinium compounds. Developments in the use of these methods in cholinesterase research were reviewed.

The Warburg experiments were mainly concerned with a critical assessment of the derivation and expression of reaction velocities for the acetylcholine-acetylcholinesterase (AcChE) reaction. The precision of these results and their compensation for potential error were investigated.

A similar investigation with the pH-stat method led to the conclusion that its use afforded advantages over the Warburg method. The pH - stat method was thus used to investigate the derivation of kinetic parameters K_M and V_{MAX}. The usefulness of the double-reciprocal plot in these derivations was indicated. The method was applied to inhibitor studies.

The benzotriazinium compounds showed anti-AcChE activity at pH 7.0 and 37°C giving p I_{50} values in the range 3.68 - 4.47 (physostigmine 7.00-7.08). Tests with selected benzotriazinium compounds indicated that inhibition was probably mixed but that interpretation of mechanism of action might be complicated by the mathematical treatment of data. Experiments with physostigmine showed that mechanism of action can alter with changes in enzyme-inhibitor incubation times.

ACKNOWLEDGEMENTS.

I wish to acknowledge, with gratitude, the interest shown and encouragement given throughout the course of the research by my supervisor, Professor A.R. Rogers.

To Dr. M.F.G. Stevens and Dr. M.S.S. Siddiqui is extended sincere thanks for the provision of the benzotriazinium compounds tested.

The interest shown in the research by my colleagues, Dr. N.C. Scott and Mr.J. Lyall is acknowledged. In particular Mr. Lyall is thanked for the use of programmes allowing the calculation of many of the results on the Olivetti 602 calculator and for his interest in the statistics of the work.

Sincere thanks is expressed to the following members of the technical staff who rendered assistance during the research; Mr. A. McK. French, Mr.J.M. Allan, Mr. R. Buchanan, Mrs. G.E. Gibson and Mr. I.J. Lamont.

To my wife, Margaret, is extended thanks for encouragement during the course of the research and for her patience and tolerance during her typing of this thesis.

CONTENTS

	page
INTRODUCTION.	1
2-Alkyl- 1,2,3-benzotriazinium compounds.	2
Aims of the present research.	9
Some factors to consider in the experimental investigation of cholinesterase activities.	10
Nomenclature and classification of enzymes.	10
Some properties of cholinesterases.	11
Physiological functions of cholinesterases.	15
A consideration of enzyme kinetics.	17
Influences of experimental variables on the activity of cholinesterases.	21
Cholinesterase inhibitors.	23
Methods for the detection and determination of cholinesterases.	27
Factors which may influence the choice of method.	28
Situation in which the method is to be employed.	29
Suitability of the method for a particular type of study.	29
Availability of apparatus and the developments of new techniques and methods.	30
Considerations of accuracy, precision, speed of determination and sensitivity in the chosen method.	31
Suitability of the method for automation.	32
Ability to alter and control variables.	33
General considerations concerning methods for detecting and determining cholinesterase.	33
Choice of methods for the present research.	35
The Warburg manometric method for the determination of cholinesterase activity.	36
The pH - stat method for the determination of cholinesterase activity.	40
EXPERIMENTAL WORK.	46
EXPERIMENTS USING THE WARBURG MANOMETRIC METHOD TO DETERMINE ACETYLCHOLINESTERASE (AcChE) ACTIVITY.	47

Contents. (continued). page

An investigation into the use of the Warburg manometric method for the determination of acetylcholinesterase activity. 48

Principle of the method. 48

Apparatus. 48

Solutions. 52

Reaction systems for enzyme-substrate studies. 53

Experimental protocol for enzyme-substrate studies. 54

Reading of manometers. 56

Flask constants. 56

Calculation of results. 57

Initial experiments involving the Warburg manometric technique for the measurement of AcChE activity. 59

Results and Discussion. 59

Further investigation into the use of the Warburg manometric technique for the measurement of AcChE activity. 68

Modification of Experimental Technique. 68

Results and Discussion. 69

An examination of the influence of some factors on the results and associated errors from the determinations of AcChE activity by Warburg manometry. 80

Results. 83

Influence of the use of compensatory 'blanks' on the results. 89

The use of the b_{30} value or the slope of the regression line relating CO_2^{30} production to time as alternative expressions of reaction velocity. 97

Experiments with inhibitors. 103

General Discussion on results from the Warburg manometric determination of AcChE activity. 107

Appendix.

Determination of reaction velocities, K_M and V_{MAX} for the interaction between AcCh and AcChE at 37^oC. 118

EXPERIMENTS USING THE pH - STAT METHOD TO DETERMINE

ACETYLCHOLINESTERASE ACTIVITY. 124

Contents (continued).

	page
An investigation into the use of the pH - stat method for the determination of acetylcholinesterase activity.	125
Apparatus.	125
Solutions.	127
Experimental Protocol.	128
Calculation of reaction velocities.	131
Results and Discussion.	133
Calculation of the kinetic parameters K_M and V_{MAX}	139
Characterisation of enzyme system under test.	142
Relationship between enzyme concentration and reaction velocity.	142
Results.	142
Relationship between enzyme activity and substrate concentration.	143
Results.	143
Influence of different batches of enzyme on the results.	146
Results.	146
Stability of Master enzyme solutions.	153
Precision of results.	155
Influence of pH on the AcCh-AcChE reaction measured by the pH - stat method at 37°C.	161
Results.	161
An investigation of some potential sources of error in the pH - stat method as applied to the reaction between AcCh and AcChE.	164
Possible errors arising from spontaneous hydrolysis of substrate.	164
Possible errors due to non-specific acid liberation by the enzyme preparation.	164
Volume errors arising from addition of titrant to achieve the required pH end-point prior to the start of the reaction.	165
Volume errors arising from the addition of titrant during the six minutes of reaction.	165

Contents. (continued).

	page
The application of the pH - stat method to the study of the AcCh-AcChE reaction in the presence of inhibitors.	167
A preliminary examination of some benzotriazinium compounds for anti-acetylcholinesterase activity.	167
Results and Discussion.	168
Experiments to ascertain the reversibility of the anti-acetylcholinesterase activity of 2-n-butyl-4-p-tolylamino - 1,2,3- benzotriazinium iodide (TBBI) at 37°C and pH 7.0.	172
Experiments to illustrate the kinetics and mechanism of the inhibition of AcChE by physostigmine.	175
Results and Discussion.	176
Experiments to illustrate the kinetics and mechanism of the inhibition of AcChE by benzotriazinium compounds.	184
(a) Experiments with 2-n-butyl-4-p-tolylamino - 1,2,3- benzotriazinium iodide.	184
Results.	184
(b) Experiments with other benzotriazinium compounds.	195
Consistency of results with TBBI as AcChE inhibitor.	200
The effect of pH on the inhibition of AcChE produced by TBBI at 37°C.	201
The effect of TBBI on the reaction between AcCh and AcChE from electric eel at pH 7.0 and 37°C.	202
General Discussion.	203
BIBLIOGRAPHY.	212

SOME ABBREVIATIONS USED IN THIS THESIS.

AcCh - acetylcholine.

AcChE - acetylcholinesterase.

anti-AcChE - anti-acetylcholinesterase.

Benzotriazinium compounds - see Table 1.

b_{30} value - corrected volume of CO_2 produced in 30 mins in Warburg manometric experiments.

df - degrees of freedom.

E - enzyme.

ES - enzyme-substrate complex.

F.F.C. - final flask concentration.

I - inhibitor.

I_{50} - concentration of inhibitor giving 50% enzyme inhibition.

p I_{50} - log reciprocal of I_{50} value.

K_M - Michaelis constant; given by $[S] = \frac{V_{MAX}}{2}$

K_i - enzyme-inhibitor equilibrium constant.

M.E.S. - master enzyme solution.

v - reaction velocity for enzyme-catalysed reaction.

V_{MAX} - maximum reaction velocity for enzyme-catalysed reaction.

γ ChE - pseudocholinesterase.

[ ]- indicates concentration.

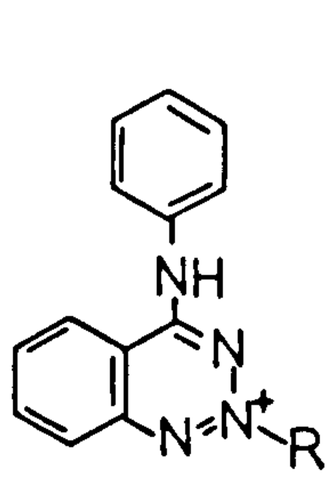
INTRODUCTION.

2 - Alkyl - 1,2,3 - benzotriazinium compounds.

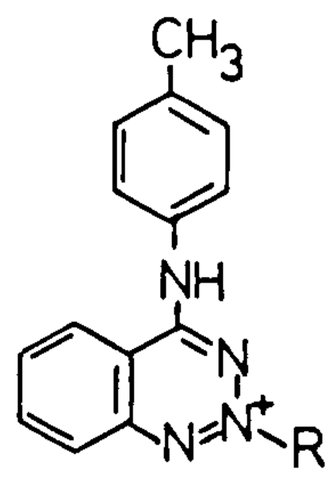
Some pharmacological properties of four homologous series of 2 - alkyl - 1,2,3 - benzotriazinium compounds were investigated by Cull (1972). The basic formulae of the four series of compounds are shown in Figure 1 and indicate the possession of a quaternary ammonium ion at the 2 - position. Such ions are often associated with pharmacological activity at cholinergic sites (see Barlow,1964). Figure 1 also shows how the four series differ in respect of the substituent in the 4 position. Both the general synthetic pathway and the elucidation of the structure of these compounds have been described by Stevens and Stevens (1970 a;b).

Table 1 gives a list of the abbreviations assigned to the compounds in connection with their pharmacological investigation.

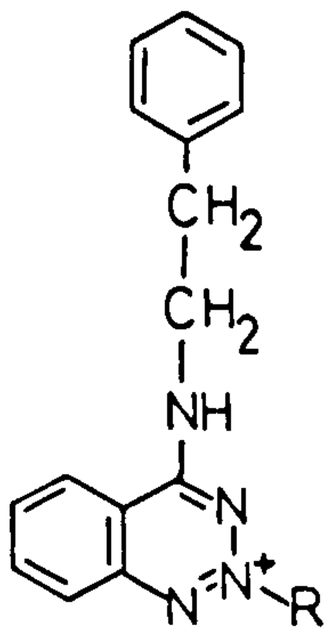
Cull (1972) showed that the benzotriazinium compounds listed in Table 1 could produce a variety of pharmacological effects on isolated skeletal muscle preparations. They could cause both initial facilitation and subsequent block of the twitch responses of the isolated rat diaphragm muscle invoked indirectly by stimulation of the phrenic nerve. The four series of compounds showed differences in respect of the degree of facilitation produced and in their ability to produce facilitation on repeated application. This facilitation was blocked by the neuromuscular blocking agents d-tubocurarine and suxamethonium. Not only could the benzotriazinium compounds block the rat diaphragm to indirect stimulation but they could also, at sub-blocking concentrations, potentiate the neuromuscular blocking activity of both d-tubocurarine and suxamethonium. Additionally physostigmine reversal of tubocurarine - induced block of the rat diaphragm was antagonised by the benzotriazinium compounds. Evidence was presented which suggested that, on the rat diaphragm preparation, benzotriazinium action might involve inhibition of acetylcholine (AcCh) synthesis in the terminal of the phrenic nerve.



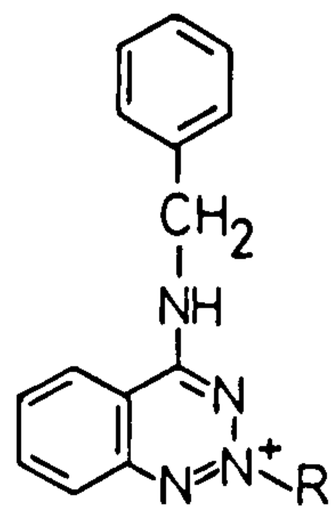
I



II



III



IV

Fig 1.

Chemical structures of the benzotriazinium compounds.

I = 4-anilino-1,2,3-benzotriazinium;
II = 4-p-tolylamino-1,2,3-benzotriazinium;
III= 4-phenethylamino-1,2,3-benzotriazinium;
IV = 4-benzylamino-1,2,3-benzotriazinium;
R = methyl, ethyl, i-propyl, n-propyl,
n-butyl or n-pentyl.
All compounds were made as the iodides.

Table 1

Abbreviations used for benzotriazinium compounds.

(From Cull,1972).

ANILINO SERIES

Substituent on Quaternary Nitrogen	Code
Methyl	AMBI
Ethyl	AEBI
n-Propyl	AnPBI
iso-Propyl	AiPBI
n-Butyl	ABBI
n-Pentyl	A5BI

p-TOLYLAMINO SERIES

Substituent on Quaternary Nitrogen	Code
Methyl	TMBI
Ethyl	TEBI
n-Propyl	TnPBI
iso-Propyl	TiPBI
n-Butyl	TBBI
n-Pentyl	T5BI

BENZYLAMINO SERIES

Substituent on Quaternary Nitrogen	Code
Methyl	BMBI
Ethyl	BEBI
n-Propyl	BnPBI
iso-Propyl	BiPBI
n-Butyl	BBBI
n-Pentyl	B5BI

PHENETHYLAMINO SERIES

Substituent on Quaternary Nitrogen	Code
Methyl	PMBI
Ethyl	PEBI
n-Propyl	PnPBI
iso-Propyl	PiPBI
n-Butyl	PBBI
n-Pentyl	P5BI

Members of all four series of compounds produced contracture of the frog rectus abdominis muscle which was not antagonised by d-tubocurarine at concentrations which would block AcCh - induced contractures. It was also shown that members of the 4-phenethylamino and 4-benzylamino series (III and IV, Fig. 1) could, after application to the muscle, cause a subsequent block of AcCh - induced contractures but that 4 - anilino and 4 - p-tolylamino derivatives (I and II Fig. 1) could not.

The benzotriazinium compounds produced a slowly developing block of the maximal twitches of the chick biventer cervicis muscle stimulated through its nerve. This block was associated with a contracture of the slow fibres of the muscle. An investigation with the 4- anilino series suggested that the compounds were acting at a post-synaptic site in this neuromuscular preparation.

Experiments were performed to examine the nature of the relationship between structural changes in the benzotriazinium molecule and potency in producing contractures in both the frog rectus and the chick biventer cervicis muscles. It was shown that, in both chick and frog preparations, in three series of compounds potency increased to a maximum with the 2-n-butyl compounds before declining with the 2-n-pentyl compounds. In the 4-p-tolylamino series the 2-isopropyl member was the most active homologue. The general order of potency between the four series was 4-p-tolylamino $>$ 4-anilino $>$ 4-phenethylamino $>$ 4-benzylamino. The results of these experiments were reported by Cull and Scott (1973). Not only was the influence on potency of the 2-alkyl group, and of the substituent on the exocyclic nitrogen at position 4, investigated but also the inter-relationships between the lipid solubilities of the compounds and potency were examined. Of further interest was the fact that in alkaline solutions the compounds tested would form an insoluble zwitterion as shown in Figure 2 .

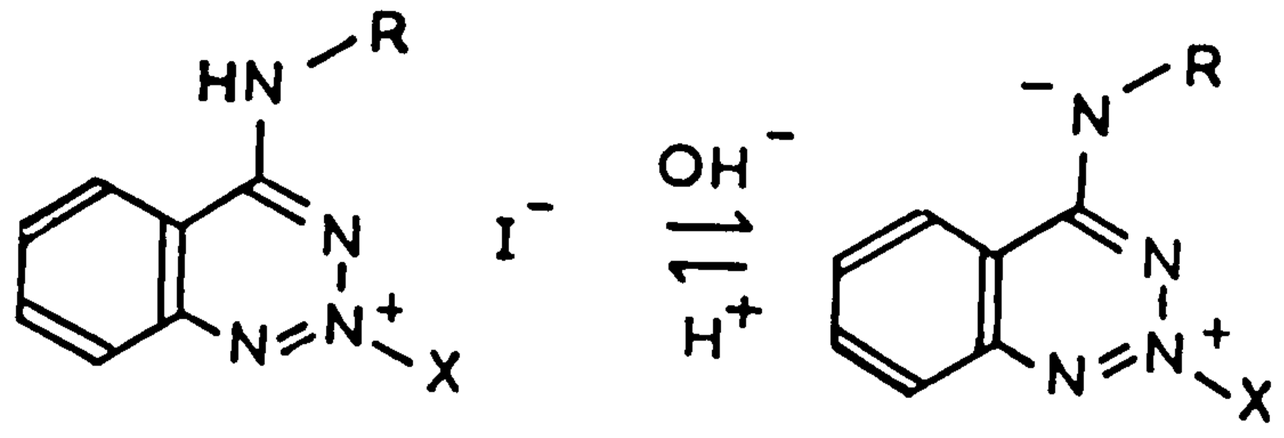


Figure 2

Formation by4substituted 2-alkyl -1,2,3-benzotriazinium compounds of zwitterion in alkaline solution.

Determinations of pK_a values for some of the compounds of the 4-anilino and 4-p-tolylamino series indicated that zwitterion formation would be negligible at pH values below 7.4. (Cull and Scott,1973). In the 4-anilino series, for compounds whose pK_a values had been determined, the percentage of compound present as zwitterion would be 3-4% at pH 7.0 and 5-7% at pH7.2. The corresponding percentages in the 4-p-tolylamino series .would be 1-3% and 2-5% respectively. Preliminary experiments have suggested that percentage zwitterion formation would be much lower in the 4-benzylamino and 4-phenethylamino series and that, when formed, the zwitterionic form of the compounds would be biologically inactive due possibly to its low solubility (Gull and Scott, 1973). A knowledge of the ionisation characteristics of these compounds is thus clearly of importance in those biological experiments in which the pH of the experiment may be above the physiological range 7.2-7.4.

It has been stated (Cull,1972) that the pharmacological effects of the benzotriazinium compounds on skeletal muscle are complex and suggest that the compounds may have more than one mode of action not only on different tissues but also on the same tissue. The resemblance of these actions on skeletal muscle to those of quinine and quinidine was also noted.

A further factor which may have influenced the activity of the benzotriazinium compounds on skeletal muscle was the ability of the compounds to inhibit cholinesterase enzymes. Accordingly the present author carried out a preliminary test on three of the compounds; the 2-methyl homologue of the 4-phenethylamino series and the 2-n-propyl homologues of both the 4-anilino and 4-benzylamino series which showed that the three compounds produced 50% inhibition of the acetylcholinesterase from bovine erythrocytes at concentrations of 75, 250 and 150 μ mol/l respectively. Physostigmine sulphate produced 50% inhibition of the same enzyme at a concentration of 0.25 μmol/l.

Cull (1972) considered that the anticholinesterase activity of these compounds was too feeble to have contributed to their ability to cause contracture of the chick biventer cervicis and the frog rectus abdominis muscles particularly as tubocurarine did not antagonise these contractures. However, in these studies, 4-anilino -2-n-propyl -1,2,3-benzotriazinium iodide was used to produce contracture of the frog rectus abdominis at a concentration of 125 μ mol/l, a concentration only half of that required to produce 50% inhibition of acetylcholinesterase. Similarly 4-benzylamino -2-n-propyl -1,2,3-benzotriazinium iodide was used on the frog rectus abdominis at a concentration (125 μ mol/l) very close to the concentration giving 50% acetylcholinesterase inhibition. On the chick biventer cervicis, too, the benzotriazinium compounds were used at concentrations which the very limited tests on acetylcholinesterase indicated might have been approximating to their 50% inhibitory concentrations. In the case of the rat diaphragm muscle, where the actions of the benzotriazinium compounds appeared to be particularly complex, Cull did consider that anticholinesterase actions of the benzotriazinium compounds might have complicated the interpretation of the results from some experiments.

It was decided, therefore, in the present research to make a more detailed study of the antiacetylcholinesterase activities of the

benzotriazinium compounds examined by Cull (1972) but under more carefully controlled conditions.

Of further interest, here, is the fact that quinine is also known to produce some degree of inhibition of cholinesterase enzymes at concentrations of approximately 0.1 to 1.0 m mol/l (Waelsch and Nachmansohn,1943; Nachmansohn and Scheenmann,1945; Wright and Sabine, 1948; Goldstein,1951). Quinidine, too, will inhibit cholinesterases (Nachmansohn and Scheenmann,1945) and is said to be a competitive antagonist selective for plasma as opposed to red cell cholinesterase (Wright and Sabine, 1948). The concentrations at which quinine and quinidine inhibit cholinesterases are similar to the concentrations of these compounds which were used by Cull for studies on skeletal muscle; a situation comparable to that of benzotriazinium compounds which are said to have actions similar to quinine and quinidine on skeletal muscle.

Aims of the present research.

The aims of the present research have been:-

1. To review the methods available for the determination of cholinesterase activity and bearing in mind their alleged precision, accuracy, and applications, to select a method, or methods, for the 'in vitro' determination of acetylcholinesterase activity in the presence of cholinesterase inhibitors.

2. To assess the limitations of the chosen method(s) by the study of the acetylcholinesterase-catalysed hydrolysis of a known substrate, acetylcholine, taking due cognisance in turn of the factors which may be influencing the measurement of such a reaction.

3. To repeat the acetylcholinesterase-catalysed hydrolysis of the selected substrate in the presence of a known acetylcholinesterase inhibitor, physostigmine, in order that a standard for acetylcholinesterase inhibition be established.

4. To apply the selected method for acetylcholinesterase determination to an investigation into the antiacetylcholinesterase activities of the 2-alkyl-1,2,3 - benzotriazinium compounds whose pharmacological actions on isolated skeletal muscle preparations had previously been studied by Cull (1972).

5. To compare the antiacetylcholinesterase activities, and if possible the mechanism of antiacetylcholinesterase activity, of the benzotriazinium compounds with that of physostigmine.

Some factors to consider in the experimental investigation of cholinesterase activities.

Nomenclature and classification of enzymes.

Some properties of cholinesterases.

Physiological functions of cholinesterases.

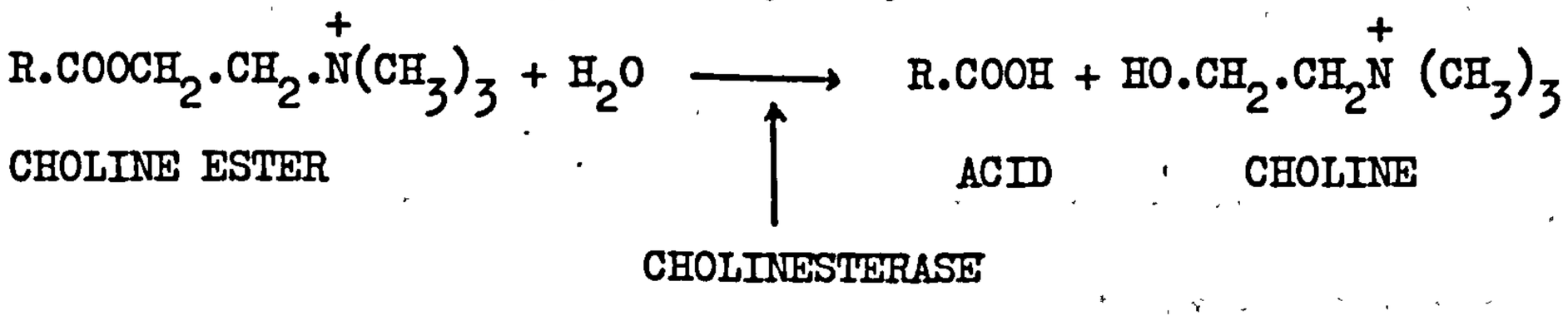
A consideration of enzyme kinetics.

Influences of experimental variables on the activity of cholinesterases.

Cholinesterase inhibitors.

Nomenclature and Classification of enzymes.

Cholinesterases have been defined by Augustinsson (1963) 'simply as enzymes which catalyse the hydrolysis of choline esters'.



In an elaboration of the above definition Augustinsson (1963) stated that:-

'Cholinesterases constitute a group of esterases which hydrolyse choline esters at a higher rate than other esters, when hydrolysis rates are compared at optimum conditions regarding substrate concentration, pH, ionic strength, etc., using preparations free from other esterases. All esterases which show this specificity are inhibited by 10^{-5}M eserine or the same concentration of neostigmine (Prostigmine), and are also much more sensitive to quaternary ammonium ions than are other esterases'.

In accordance with the systematic numbering scheme for enzymes given in the Report of the Commission on Enzymes of the International Union of Biochemistry (Anon, 1961) and in the Recommendations (1964) of the International Union of Biochemistry on Enzyme Nomenclature (Anon, 1965) esterases were classified amongst the hydrolases (Classification 3). Hydrolases acting on ester bonds were given the classification 3.1,

the various cholinesterases being classified amongst the carboxylic ester hydrolases and numbered in the 3.1.1. series.

Table 2 lists the classifications and nomenclature of the cholinesterases and related enzymes (Anon., 1965) showing that cholinesterases included enzymes classified under the numbers 3.1.1.7; 3.1.1.8 and 3.1.1.9. The use of other names (Table 2) for these enzymes has shown variations and was not recommended (Anon, 1965).

The ways in which the various types of cholinesterase can be distinguished from each other and from the related enzymes listed in Table 2 include differences in their behaviour towards substrates and inhibitors. Some of these differences are presented in Table 3. The cholinesterases have been subdivided according to their preferred substrates; that is the substrate which they hydrolyse most rapidly. For example the acetylcholine (AcCh) - preferring enzyme is termed acetylcholinesterase (AcChE) whilst the enzyme for which benzoylcholine (BzCh) is the preferred substrate was termed benzoylcholinesterase (BzChE) (Usdin,1970).

Sometimes the cholinesterases are divided simply into AcChE and pseudocholinesterase (ψ ChE). The latter term covers cholinesterases for which AcCh is not the preferred substrate and has included butyrylcholine esterase - preferred substrate butyrylcholine, propionylcholinesterase - preferred substrate propionylcholine and BzChE (Usdin, 1970).

The evolution of the nomenclature applied to cholinesterases and related enzyme was discussed by Augustinsson (1963) and Usdin (1970).

The Recommendations (1972) of the Commission on Biochemical Nomenclature (Enzyme Nomenclature' (1972); Amsterdam: Elsevier,1973) discontinued the classification, 3.1.1.9, and included BzChE in the classification 3.1.1.8.

<u>Some properties of cholinesterases.</u>

A distinction between AcChE and ψ ChE's is that in general the

Table 2

Classification of the cholinesterases and some related enzymes

(From "Enzyme Nomenclature"; Anon., 1965)

Number.	Systematic name.	Recommended trivial name	Other names.	Reaction.
3. Hydrolases.				
3.1. Acting on ester bonds.				
3.1.1. Carboxylic ester hydrolases.				
3.1.1.1	Carboxylic-ester hydrolase.	Carboxylesterase	Ali-esterase B-esterase.	A carboxylic ester + H_2O = an alcohol + a carboxylate.
3.1.1.2	Aryl-ester hydrolase.	Arylesterase.	A-esterase paraoxonase.	A phenyl acetate + H_2O = a phenol + acetate.
3.1.1.3	Glycerol-ester hydrolase.	Lipase		A triglyceride + H_2O = a diglyceride + a fatty acid ion.
3.1.1.6	Acetic-ester hydrolase	Acetylesterase	C-esterase.	An acetic ester + H_2O = an alcohol + acetate.
3.1.1.7	Acetylcholine hydrolase.	Acetylcholinesterase	True cholinesterase.	Acetylcholine + H_2O = choline + acetate.
3.1.1.8	Acylcholine acylhydrolase.	Cholinesterase.	Pseudocholinesterase.	An acylcholine + H_2O = choline + an anion.
3.1.1.9 *	Benzoylcholine hydrolase.	Benzoylcholinesterase.		Benzoylcholine + H_2O = choline + benzoate.

* Classification now discontinued (see Page 11).

Table 3

Some ways in which certain carboxylic ester hydrolases (esterases) including cholinesterases may be differentiated.

Esterases inhibited by organophosphorus compounds includes -			Esterases resistant to organophosphorus compounds.	
Cholinesterases	Carboxylesterases	Lipases	Arylester hydrolases (A - esterases).	Acetic-ester hydrolase (Acetylesterase)
Hydrolyse choline esters faster than other esters under optimum conditions.	E.C.3.1.1.1. Unable to hydrolyse choline esters. Hydrolyse short-chain aliphatic carboxyesters.	E.C.3.1.1.3	E.C.3.1.1.2 Hydrolyse aromatic esters at a high rate. -SH enzymes, inhibited by chelating agents and heavy metal ions (Hg^{++}) May cause hydrolysis of some organophosphorus esters (e.g.D.F.P).	E.C.3.1.1.6 Preferential action on acetic esters. Resistant to chelating agents and heavy metals (Hg^{++}).
Inhibited by 10^{-5}mol/l eserine.	Most resistant to 10^{-5}mol/l eserine.			
Include - Acetylcholinesterase E.C.3.1.1.7. Cholinesterase E.C.3.1.1.8.				

former shows inhibition with excess substrate whilst the latter do not. In their reviews concerning cholinesterases Augustinsson (1963), Cohen and Oosterbaan (1963) and Usdin (1970) discussed the questions of substrate specificity and substrate inhibition. Where there is inhibition of an enzyme by excess substrate an optimum substrate concentration for that enzyme will exist. In respect of AcChE the optimum concentration of AcCh was generally said to lie in the range 2.5 m mol/l to 3 m mol/l (Cohen and Oosterbaan,1963). Nabb and Whitfield (1967) compared the relationships between AcCh concentration and both plasma cholinesterase activity and erythrocyte AcChE activity in humans, dog, rabbit and rat, finding somewhat lower optimum concentrations (0.88 to 1.76 m mol/l) for AcCh in respect of the red cell AcChE than those quoted before. By contrast Goyer (1968) found an optimum AcCh concentration of approximately 5 m mol/l for the cholinesterase of rat skeletal muscle whilst Coppage (1971) showed that 10 m mol/l was the approximate optimum AcCh concentration for fish brain AcChE.

Usdin (1970) reviewed many of the properties of cholinesterases which could influence their activity and stated that, despite certain generalised differences between them, there was no sharp line of demarcation between the properties of γ ChE and AcChE. He pointed out the marked differences in cholinesterases from different species or from different organs within the same animal having regard to their reactions with particular substrates. Further he reviewed the wide variety of species and tissues reported as having cholinesterase activity and stated that levels of cholinesterase activity may vary from species to species and organ to organ and may also vary from strain to strain within a species; between male and female, and between newborn and adult. The complication of the existence of genetically determined variants of γ ChE having different enzymatic activities was also considered. Purification of cholinesterases, with attendant alterations in enzyme activity and the commercial availability of

cholinesterases of both the AcChE and γ ChE type were further points covered by this review. Sources for commercial AcChE include the electric organ of electric eel and bovine erythrocytes whilst γ ChE may be obtained commercially from horse serum.

Berman (1973) has shown that it is possible for AcChE to show considerable differences in structure when enzyme from erythrocytes is compared with that from electric eel despite general similarities in the kinetic properties of the enzymes.

It will be clear from the foregoing discussion that the properties of a cholinesterase may have a profound effect on the qualitative and quantitative results obtained in experiments with the enzyme. Thus care should be exercised in the selection and reporting of the cholinesterase used in a given experiment whilst, in turn, the choice of enzyme may be governed by the requirements of the experiment.

Physiological functions of cholinesterases.

A further distinction between AcChE and γ ChE may be made in respect of their physiological functions.

Amongst reviews relating to the physiological functions of AcChE may be mentioned those of Koelle(1963), Karczmar (1967) and Usdin (1970). AcChE is known to be involved in the hydrolysis of AcCh at cholinergic synapses both in the nervous system and at neuroeffector junctions.

This enzyme, as part of the AcCh/AcChE system, has also been implicated in the process of axonal conduction (Nachmansohn,1959;1963 and 1966). However, Ehrenpreis, Fleisch and Mittag (1969) discussed the evidence against involvement of an AcCh receptor system in axonal conduction and concluded that such a receptor system was not present in the nerve axon.

Ehrenpreis *et al* (1969) have also considered the claims that cholinesterase, particularly AcChE, and the cholinergic pharmacological tissue receptor have a common identity. Attention was drawn to two concepts. One was that a small fraction of the active sites on the

enzyme, when incorporated into membranes, represent cholinergic receptor molecules. The second was the possible importance of allosteric sites on the enzymes in the binding of cholinergic agents. Investigations into this concept have continued. For example Kato, Yung and Ihnat (1970) in a nuclear magnetic resonance study on AcChE binding sites concluded that it remained to be shown whether or not AcChE functioned, physiologically, as the post-synaptic receptor for AcCh. Zupancic (1970) presented results of a kinetic study which suggested that the anionic centres of AcChE, built into excitable membranes of mouse diaphragms, were identical with nicotinic cholinoreceptive sites on the same macromolecule. As a result of experiments on the ventricle of frog heart Zupancic, Majcen and Stalc (1972) stated that AcChE was identical with the muscarinic cholinoreceptive protein. In turn Jain, Mehl and Cordes (1973) considered that AcChE may be able to function in the same way as the cholinergic receptor with regard to the formation of ion-conducting channels in membranes.

Involvement in the control of membrane permeability has also been proposed as a physiological function of AcChE (see Koelle,1963).

In contrast to AcChE, a physiological function for ψ ChE is less clear. The many varying proposals concerning ψ ChE have again been reviewed by Usdin (1970). These include protection of AcChE against inhibitors; protection of the body against BuCh; maintainance of the correct choline/AcCh ratio in the plasma; involvement in slow nerve conduction processes; involvement in lipid metabolism and in the control of membrane permeability.

It may be that some of the proposals are linked by factors in common. Clitherow, Mitchard and Harper (1963) offered the suggestion that ψ ChE may protect the body against BuCh formed under the influence of the butyryl co-enzyme A produced by lipogenesis. Again, Ballantyne (1968a) discussed the possible role of cholinesterases in the

metabolism of lipids and considered that BuChE by acting on the lipid component of the cell membrane might regulate cell membrane permeability for the transport of cations.

Work continues in order to elucidate the possible physiological functions of ψ ChE. For example Heffron (1972) investigated the possibility that the enzyme might play a contributory role in the transmission process at the neuromuscular junction of the rat diaphragm.

It follows that either enzyme studies involving the known or possible physiological functions of cholinesterases or investigations into the mechanisms of the pharmacological actions of drugs which interact with cholinesterases will require care in the selection of the type of enzyme used in the study.

A consideration of enzyme kinetics.

There have been many texts of various degrees of comprehensiveness, dealing with enzyme kinetics. They include those of Dixon and Webb (1964) and Zeffren and Hall (1973) which form the basis of the following discussion.

Dixon and Webb (1964) stated that 'The characteristic property and function of enzymes is the catalysis of chemical reactions. Any fundamental study of this catalytic function must be based on quantitative measurements of the rate of the catalysed reaction.' They pointed out that the progress curves of most enzyme catalysed reactions show a reduction of reaction velocity with time and gave reasons for this reduction. These include the accumulation of reaction products which inhibit the enzyme or make the reverse reaction more important and a fall in the degree of saturation of enzyme with substrate. Therefore study of enzyme reactions usually involves measurement of the initial reaction velocity from the first part of the progress curve before the reaction rate has fallen off and where the conditions of the reaction are accurately known. In this thesis 'reaction velocity' will,

unless otherwise specified, refer to the initial reaction velocity. Variation should be made by only one factor influencing the reaction velocity at a time.

The satisfactory estimation of enzyme activity requires conditions in which a linear relationship exists between the reaction velocity and enzyme concentration under constant assay conditions. Such a relationship would show, for example, that the enzyme preparation did not contain inhibitors or activators whose effects were seen at high enzyme concentrations and that substrate concentration was greatly in excess of enzyme concentration. It would also show that the test method was not limited in its ability to measure reaction velocity over the given enzyme concentration range.

There generally exists a relationship between initial substrate concentration and reaction velocity which is represented by a rectangular hyperbola. This is seen with a given initial enzyme concentration when the initial concentration of a single substrate only is allowed to alter. In the case of some enzymes (e.g. as described for AcChE.p14)excess substrate may inhibit the enzyme.

Studies of enzyme kinetics thus require careful consideration of enzyme and substrate concentrations, time, experimental variables and the initial concentrations of any inhibitors, activators or co-factors present.

The enzyme (E) - substrate (S) reaction was described by Michaelis and Menten (1913) in the terms of a two stage reaction:-

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E$$

ES is an enzyme substrate complex breaking down to reaction products (P) with regeneration of free enzyme. k_1, k_{-1} and k_2 are rate constants for the separate reactions.

The assumption of Michaelis and Menten was that equilibrium between E and S was attained so rapidly in relation to the breakdown

of ES that ES always remained in equilibrium with E and S during the reaction. Thus $k_{-1} \gg k_2$ and the equilibrium constant for the dissociation of ES was given by $\frac{k_{-1}}{k_1} = K_s$.

This assumption, in many cases, may not hold and the reaction may be more appropriately described by the steady state theory of Briggs and Haldane (1925). Here the rates of formation and breakdown of ES, at a given moment were regarded as essentially equal. The concentration [ES] was considered to be constant over the period of measurement of reaction velocity, long term changes in [ES] with fall in [S] being neglected.

Short term steady state conditions are reflected by the Michaelis equilibrium constant K_M where $K_M = \frac{k_{-1} + k_2}{k_1}$. Therefore K_M and K_s are identical only under certain circumstances.

It should be recognised that many enzyme - substrate reactions are thought to be more complex than the two stage reaction described. Some of the proposals for cholinesterase-catalysed reactions were described by Usdin (1970) and included the situation where an acyl-enzyme (EA) intermediate is formed:-

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P$$

The reaction velocity (v) of the enzyme-catalysed reaction is given by:-

$$v = \frac{V_{MAX.}\,[S]}{K_M + [S]} \qquad (1)$$

(See Dixon and Webb, 1964)

V_{MAX} is the maximum reaction velocity.

Factors may alter reaction rate by affecting the rate of formation of [ES] as expressed in K_M, or the rate of product formation expressed by V_{MAX}. Experimental determinations of K_M and V_{MAX} will thus give kinetic parameters which characterise the enzyme-catalysed reaction under the stated conditions. Zeffren and Hall (1973) emphasized that

whilst kinetics are a valuable tool in the elucidation of the mechanisms of enzyme-catalysed reactions they cannot lead to a unique interpretation of a reaction mechanism but rather provide a rate law with which several mechanisms may be consistent.

Problems of interpretation of results may be increased in the 'in vivo' situation. Gillette (1971) has pointed out the assumptions in the Michaelis-Menten equations. These include the assumption that the total concentrations of substrate and inhibitors represent the free concentrations of these substances near the active site of the enzyme; that the products of reaction are released rapidly from the enzyme; and that only one substrate molecule is bound to an enzyme site. The presence of diffusion barriers around the enzyme site and the binding of reagents to tissue protein 'in vivo' or the presence of potent inhibitors in small concentrations relative to enzyme concentration may invalidate these assumptions.

From equation 1 it will be seen that when v is half maximal $(\frac{V_{MAX}}{2})$ then $[S] = K_M$. A graphical plot of v against [S] would yield a hyperbolic curve on which the value of [S] corresponding to $v = \frac{V_{MAX}}{2}$ would give the value of K_M. Because of the practical difficulty of ensuring the V_{MAX} has, in fact, been reached linear transformations of equation 1 or statistical treatments of experimental data have been employed for the measurement of K_M and V_{MAX} (Dixon and Webb, 1964).

The plot of $\frac{1}{v}$ against $\frac{1}{[S]}$ (Lineweaver and Burk, 1934) for instance utilised a re-arranged form of equation 1 in which $\frac{1}{v} = \frac{K_M}{V_{MAX}} \cdot \frac{1}{[S]} + \frac{1}{V_{MAX}}$. A linear graph results having a slope of $\frac{K_M}{V_{MAX}}$ and intercepts of $\frac{1}{V_{MAX}}$ & $\frac{-1}{K_M}$ on the ordinate and abscissa respectively.

Alternative linear rearrangements of equation 1 include

$$v = V_{MAX} - K_M \cdot \frac{v}{[S]}$$

$$\text{and } \frac{[S]}{v} = \frac{K_M}{V_{MAX}} + \frac{1}{V_{MAX}} \cdot [S]$$

In the first of these cases v may be plotted against $\frac{v}{[S]}$ to give a line with a slope equal to $-K_M$ and an intercept equal to V_{MAX} on the ordinate (Hofstee,1959). In the second case the line resulting from a plot of $[\frac{S}{v}]$ against [S] has a slope of $\frac{1}{V_{MAX}}$ and an intercept on the abscissa of $-K_M$ (Hanes,1932).

There are claims that the estimate and reliability of K_M and V_{MAX} may be influenced by the linear transformation chosen (Dowd and Riggs, 1965; Colquhoun 1969).

Influences of experimental variables on the activity of cholinesterases.

Dixon and Webb (1964) reviewed the effects that pH, the presence of inorganic ions and temperature might have on enzyme activity in general. Cohen and Oosterbaan (1963) in turn reviewed the effects of these variables on the interaction between AcChE and substrates. They pointed out how pH might influence the degree of dissociation of ionizing groups both on the substrate and on the enzyme and this might influence the activities of these entities. Results were quoted, for instance, which gave the optimum pH range (between 8 and 9) for the maximum activity of AcChE from the eel as measured by its ability to hydrolyse AcCh. The activating effect that certain bivalent (Ba^{++},Ca^{++}, Mg^{++}, and Mn^{++}) and univalent (Na^+ K^+, Li^+) cations might have on AcChE was also considered and an optimum ionic medium of 0.15 mol/l NaCl + 0.01 to 0.04 mol/l $Mg\ Cl_2$ recommended for experiments involving interaction between AcChE and substrates.

However the effect of inorganic ions is not straightforward. Ivanova (1967) showed that NaCl might either activate or inhibit erythrocyte AcChE depending on the relative concentration of NaCl and AcCh substrate. A similar finding relating to AcChE was reported by Brestkin and Ivanova (1970) in respect of LiCl and $CaCl_2$ whilst Brestkin, Brik, Volkova,Maizel and Rozengart (1970) showed that certain concentrations of KCl could cause a reduction in the reactivity of both

equine blood serum cholinesterase and bovine erythrocyte AcChE towards AcCh. The depressant effect that the HCO_3^- ion might have on cholinesterase activity (Smallman and Wolfe,1954) is of particular relevance to the Warburg manometric assay of this enzyme discussed elsewhere in this thesis.

It is also known that inorganic ions may alter the influence of other compounds on cholinesterase activity. Changeux(1966) showed that the ionic strength of the reaction medium could reduce the affinity of AcChE for both substrate and some reversible competitive inhibitors. Roufogalis and Thomas (1968 a,b) demonstrated that a medium of NaCl and $MgCl_2$, in certain concentrations, could both reduce the potentiation of the AcChE catalysed hydrolysis of AcCh effected by certain quaternary ammonium compounds and modify the influence of tetra-alkylammonium salts on the activity of AcChE with respect to phenyl acetate as substrate. The importance of both substrate concentration and ionic strength in regard to the action of another compound on cholinesterase has again been demonstrated by Crone (1973) in relation to the action of gallamine on AcChE. He also showed that ionic strength could influence the recovery of activity of phosphorylated enzyme.

A need to consider the influence that ionic strength may have both on the conformation of cholinesterase inhibitor molecules and also on enzyme structure was pointed out by Changeux(1966) whilst Crone (1973) stated that cholinesterase may aggregate in media of low ionic strength, the aggregated enzyme having low esteratic activity.

The influence that temperature changes can have on cholinesterase activities was discussed by Witter (1963) who pointed out the need for careful control of the reaction temperature in order to achieve accurate results from cholinesterase determinations.

A need for the careful control of the pH and ionic composition of cholinesterase reaction media will also be apparent. That the

variables affecting cholinesterase determinations may present problems to the clinician has been pointed out by Hunter (1970).

Cholinesterase Inhibitors.

The types of inhibitor which may influence the activities of enzymes have been described by Dixon and Webb (1964). They point to the basic division of inhibitors into reversible and irreverible types. Reversibility implies a spontaneous return of activity on removal of the inhibitor, a situation different from reactivation in which free enzyme is regenerated by chemical reaction with the inhibited enzyme. In turn enzyme inhibition is distinguished from chemical destruction of enzyme.

Reversible inhibitors exist in equilibrium with the enzyme the enzyme-inhibitor dissociation constant, K_i, being related to the affinity between enzyme and inhibitor, is thus a measure of inhibitor activity. It is often termed the inhibitor or inhibition constant. Here the inhibitor concentration governs the degree of inhibition achieved.

Irreversible inhibitors, by contrast, show a progressive inhibition with time, the inhibition becoming complete provided that inhibitor concentration is in excess of enzyme concentration. Inhibitor activity, here, is represented by a rate constant.

Dixon and Webb (1964) showed how reversible inhibition may be competitive, non-competitive or mixed, the type of inhibition being judged in kinetic terms by the effect of the inhibitor on both the Michaelis constant, K_M, and the maximum reaction velocity, V_{MAX}, of the corresponding enzyme-substrate reaction in the absence of inhibitor.

Competitive inhibitors effectively compete with the substrate for the enzyme, thus V_{MAX} can be achieved if substrate concentration is sufficiently high relative to inhibitor concentration. Further the inhibitor will effectively reduce the affinity between enzyme and substrate and thus increase K_M. The extent of this increase is related to K_i for the particular enzyme-inhibitor complex.

It is considered that in non-competitive inhibition the binding of inhibitor to enzyme is such that it does not interfere with substrate-enzyme binding. Hence K_M is not affected. The inhibitor, however, serves to reduce V_{MAX} by a factor related to K_i by altering the rate of formation of the products of the enzyme-catalysed reaction.

The above discussion represents the simplest classification of inhibitors. Dixon and Webb (1964) clearly illustrated situations in which inhibitors may exert either partially competitive or partially non-competitive actions. The kinetic equations, in thsse cases, differ from those representing the fully competitive or fully non-competitive situation.

Additionally mixed types of inhibition are seen in which an inhibitor may affect both K_M and V_{MAX}.

It is possible to assess the type of inhibition produced by a compound by graphical treatment of experimental results. The experiments may involve variation in substrate concentration at fixed inhibitor and enzyme concentrations. Alternatively experiments may involve variable inhibitor concentrations but fixed substrate and enzyme concentrations. From such experiments K_i may be readily derived graphically in cases of fully competitive or fully non-competitive inhibition (Dixon and Webb 1964).

It should be noted that the kinetics of enzyme-inhibitor reactions may well change with an alteration in the reaction conditions (Stein and Lewis,1969).

There is an extensive literature on the inhibitors of cholinesterases. In 1963 the Handbuch der Experimentellen Pharmakologie (ed.Koelle,1963) contained numerous papers which illustrated three main areas of interest in anticholinesterase (anti-ChE) research. These areas were concerned with:-

(a) The chemical classification and biochemical reactions of anti-ChE's.

(b) The systematic pharmacology of anti-ChE's which is linked with the functioning of the body cholinergic systems and

(c) The toxicology and therapeutic applications of anti-ChE's.

The first of these areas covered not only the active site of cholinesterases (Cohen and Oosterbaan,1963) but also the structure-activity relations amongst anti-ChE's (Long,1963; Holmstedt,1963) and the metabolism of organophosphorus inhibitors. This latter topic (Mounter,1963) was of concern because of the use of these potent compounds as pesticides and potentially as chemical warfare agents, pharmacological investigation tools and in medicine. Hence any modification of potency as a result of metabolism may affect their use.

The consideration of the toxicology and therapeutic applications of anti-ChE's included a contribution on the reactivation of phosphorylated AcChE (Hobbiger,1963), again a reflection on the potential toxicological hazards of organophosphorus anti-ChE's.

Development of cholinesterase reactivators and their mechanisms of action formed part of the review on AcChE by Engelhard, Prchal and Nenner (1967). In turn Way and Way (1968) discussed the metabolism of these reactivators including the potential toxicological implications due to cyanide liberation during their metabolism.

Karczmar (1967b) reviewed the pharmacological, toxicological and therapeutic properties of anti-ChE agents. The role of anti-ChE's in investigations of possible central cholinergic mechanisms was discussed as were the experiments with anti-ChE's. which have led to knowledge of aspects of the physiology and pharmacology of the neuromuscular junction, the autonomic ganglia and autonomic effector sites. Therapeutic uses to which anti-ChE's had been put included use as miotics; employment in situations requiring an increase in smooth muscle tone; counteraction of the effect of curare-like muscle relaxants used in conjunction with anaesthetics and use in the diagnosis and treatment of myasthenia gravis, a condition associated with skeletal muscle weakness.

Structure-activity relationships amongst cholinesterase inhibitors, and the light that such studies might shed on the structural features of cholinesterase receptor sites constituted part of a text on relationships between chemical structure and pharmacological activities by Cavallito (1968).

A further comprehensive treatise on anti-ChE agents may be mentioned (ed. Karczmar,1970). Its Introduction gave the history of the anti-ChE's, including the role of studies on physostigmine in this history. The other major topics dealt with were the reactions of cholinesterases (Usdin,1970) and the toxicity of anti-ChE's (Wills,1970).

Recent reviews concerning cholinesterase inhibitors as pesticides include those published by the World Health Organisation (1971) and the review by Corbett,(1974).

It will be seen that both established inhibitors of cholinesterase and compounds newly discovered to possess this type of action may have a usefulness in situations as diverse as the experimental laboratory, the clinic, and as pesticides.

Methods for the detection and determination of cholinesterases.

Methods for the detection of cholinesterases and determination of cholinesterase activity have been reviewed on numerous occasions. Some reviews (Nachmansohn and Wilson,1955; Witter,1963; Augustinsson,1957; 1971) have been devoted primarily to such methods whilst in others these methods have been part of a more comprehensive survey of cholinesterases (Augustinsson, 1963; Usdin,1970).

Augustinsson (1971) classified available methods into four main types based on the hydrolysis of choline, or thiocholine, esters as substrates under the influence of cholinesterases:-

$$(CH_3)_3\overset{+}{N}CH_2CH_2O\,CO\,R + H_2O \longrightarrow (CH_3)_3\overset{+}{N}CH_2CH_2OH + HO\,CO\,R$$

Choline ester — Choline — Acid

$$(CH_3)_3\overset{+}{N}CH_2CH_2S\,CO\,R + H_2O \longrightarrow (CH_3)_3\overset{+}{N}CH_2CH_2SH + HO\,CO\,R$$

Thiocholine ester — Thiocholine — Acid

1. Methods based on the chemical determination of the disappearance of ester by measuring that ester remaining at given times after the start of the reaction.
2. Methods based on the production of choline.
3. Methods based on the production of thiocholine.
4. Methods based on the production of acid.

Additionally other methods were reviewed which employed non-choline esters as substrates.

Cholinesterase activity was originally determined by biological assays involving incubation of cholinesterase with AcCh for a given period of time before stopping the hydrolysis and estimating the residual AcCh pharmacologically (see Augustinsson 1957). The pharmacological estimation of the AcCh might be performed on a variety of tissues including the longitudinal muscle of the leech and the frog rectus abdominis muscle (Chang and Gaddum,1933). Such methods were replaced by more convenient and accurate chemical determinations of activity although relatively recently a microbiological determination

of choline formation based on the growth-promoting action of this compound in a choline - less mutant of the fungus Neurospora crassa was proposed (Schatzberg-Porath, Zahavy and Gitter, 1963). Biological tests for cholinesterase activities may still be of use. Heffron (1972) for instance made use of the frog rectus abdominis muscle to determine the AcCh and BuCh concentrations of solutions as part of an assessment of cholinesterase levels in rat diaphragm muscle treated with a selective organophosphorus cholinesterase inhibitor.

The experimental techniques involved in chemical determinations of cholinesterases vary widely. Methods involving measurement of acid production have included gasometric techniques, of which the best known example is the Warburg manometric technique (Ammon,1933); measurement of pH change with time (Michel,1949); titration of the released acid with alkali to a fixed end point (Stedman, Stedman and Easson, 1932) and radiometric estimations of acetic acid - ^{14}C (Reed,Goto and Wang, 1966). Measurement of ester disappearance has involved colorimetric (Hestrin,1949); ultraviolet spectrophotometric (Kalow and Lindsay,1955) and radiometric techniques (Winteringham and Disney,1964). In turn thiocholine production has been estimated polarographically (Kramer, Cannon and Guilbault,1962); iodometrically (Augustinsson,1955a) and by colorimetry (McOsker and Daniel,1959; Ellman,Courtney, Andres and Featherstone,1961).

<u>Factors which may influence the choice of method.</u>

Selection of a method is not without difficulty. Amongst factors which might be considered when making the choice are the following:-

1. Situation in which the method is to be employed.
2. Suitability of the method for a particular type of study.
3. Availability of apparatus and the developments of new techniques and methods.
4. Considerations of accuracy, precision, speed of determination and sensitivity in the chosen method.

5. Suitability of the method for automation.

6. Ability to alter and control variables.

1. Situation in which the method is to be employed.

Witter (1963) discussed the suitability of methods for use in field tests for cholinesterase in which there is a requirement for methods which are easy to perform with simple equipment and with a minimum of manipulations and reagents. He also indicated ways in which the techniques for the collection of blood samples, their transportation from the field, and the handling of samples in the laboratory could influence the subsequent cholinesterase determinations. Problems peculiar to the determination of cholinesterase in autopsy specimens were also discussed. Augustinsson and Holmstedt (1965) examined the collection of blood samples in the field under tropical conditions by collecting such samples on filter paper prior to laboratory estimations for cholinesterase activity.

2. Suitability of the method for a particular type of study.

Some methods are more suitable for certain investigations than are other methods. The automated recording technique for titrating acid released by cholinesterase-catalysed hydrolysis of substrates, the so-called pH - stat method, was said to be of great value for studying the kinetics of the initial stages of such reactions as rate curves can be produced in as little as two minutes (Witter,1963 ; Augustinsson, 1971).

The histochemical techniques involved for the cytological localisation of cholinesterases were outlined by Koelle (1963) and Eränkö (1967). Many of these techniques employ non-choline esters as substrates which when hydrolysed give reaction products easily detected by colorimetric or other methods. In the use of non-choline ester substrates the specificity of the enzyme under study for the particular substrate should be known (Augustinsson, 1971).

A radiometric method capable of detecting both cholinesterase and choline acetylase activity simultaneously in single tissues was

employed by Buckley and Heaton (1970). The suitability of radiometric determination for the 'in situ' assay of cholinesterases in intact tissues to which AcCh had been externally applied in connentrations as low as nanomolar has been described by Ehrenpreis, Mittag and Patrick (1970). This latter study stemmed from concern with the use of 'in vitro' tests employing relatively large substrate concentrations to gain ideas as to the nature of events at cholinergic sites 'in vivo'.

A final example of the suitability of a particular method of cholinesterase determination for a particular type of study is the claim that the colorimetric method of Ellman et al (1961) can be used as part of a simple demonstration designed to illustrate to students the nature and fundamental properties of enzymes (Fried and Howse,1971).

3. Availability of apparatus and the developments of new techniques and methods.

Of the methods in use for cholinesterase determinations some, for example the automated pH - stat and the Warburg manometric methods, require the provision of relatively elaborate apparatus (see pages 36,40 etse a factor which may impose a restraint on the choice of method.

By contrast the availability of new experimental techniques may lead to new methods which may, in turn, lead to applications in which the determination of cholinesterase activity may be of value. A comparison of the reviews of Augustinsson (1957; 1971) reveals that the latter contains descriptions of the polarographic method of Kramer et al, (1962) and of the colorimetric method of Ellman et al (1961) both for the detection of thiocholine production and both methods developed since the time of the first review. Gas chromatography (Cranmer and Peoples, 1971) has been applied to the determination of plasma and red blood cell cholinesterase whilst Kato (1968;1972a;1972b) and Kato et al (1970) have employed nuclear magnetic resonance spectroscopy to study serum cholinesterase kinetics and to investigate the binding sites of acetylcholinesterase. Baum (1971) reported the use of a liquid membrane

electrode having a high selectivity for AcCh over choline and various inorganic cations and which thus allowed a continuous electrochemical determination of the rate of change of substrate ooncentration in the presence of the active enzyme. This method was subsequently applied to a kinetic analysis of the interaction of blood cholinesterases with three choline esters (Baum, Ward and Yauerbaum, 1972). Gel electrophoresis has been used for separating cholinesterase isoenzymes prior to their assay by spectrophotometric scanning, a technique permitting study of the kinetics of enzyme catalysis and inhibition (Chiu, Tripathi and O'Brien, 1972). Recently Goodson, Jacobs and Davis (1973) have immobilised horse serum cholinesterase on the surface of open pore urethane foam and used the resulting product for the monitoring of air and water for the presence of insecticide enzyme inhibitors.

4. Considerations of accuracy, precision, speed of determination and sensitivity in the chosen method.

Augustinsson (1963) classified the precision of methods recommended for the assay of cholinesterases as high (2-3%), moderate or low, the Warburg manometric and pH - stat methods being considered the methods of high precision. Indications of the precision and accuracy of various methods were again given by Augustinsson (1971). Here he claimed that the Warburg manometric technique was one of the most accurate and reliable methods for assaying cholinesterase, the accuracy being possibly of the order of $\pm$ 2%. Additionally the titrimetric method was stated to be one of the most convenient and precise methods known whereas, by contrast, field tests involving indicator methods probably have an error of 10 - 25%.(Augustinsson, 1971; see also Witter, 1963).

It is likely that claims for different assay methods will vary as the methods are modified. Usdin (1970) stated that amongst reasons for modifying techniques is the attempt to increase sensitivity and

speed of assays particularly in the study of reversible inhibitors where there is a necessity to maintain steady inhibitor and substrate concentrations during the course of the assay. Mention has already been made of the radiometric method of Ehrenpreis et al (1970) for the 'in situ' assay of cholinesterases, the method being sensitive to nanomolar concentrations of AcCh. Buckley and Nowell (1966) used a spectrophotometric method for the microcolorimetric determination of cholinesterase activity in rat diaphragm motor end plates which detected cholinesterase activity of the order of 10^{-11} moles substrate hydrolysed per end plate per hour. The colorimetric method of Ellman et al (1961) is also said to be sensitive, requiring blood samples of the order of only 10 µl for assay purposes; similarly Aldrich, Walker and Patnoe (1969) modified the pH - stat method to handle capillary tube samples for blood cholinesterase assay whilst Uete, Tsuchikura and Hoshida (1970) reported a fluorimetric assay of blood cholinesterase requiring less than 0.05 ml of serum or plasma.

5. Suitability of the method for automation.

The automation of a method of determination can greatly increase the convenience in the use of that method. The description by Nielands and Cannon (1955) of automatic recording pH instrumentation led to the use of automated pH - stat equipment in the measurement of cholinesterase activity. Other methods for determining cholinesterase activity have also been automated some of which are described by Witter (1963) and Augustinsson (1971). An example of the use of an automated method may be seen in the use by Stein and Lewis (1969) of a system based on the principle of continous flow dialysis for the investigation of the inhibition by physostigmine of acetylcholinesterase. Here the acetic acid produced by enzyme - substrate reaction in the presence of physostigmine, sampled from a programmed turntable, was fed into a dialyzer where the acid was picked up in an indicator stream and analysed colorimetrically.

A further example is seen in the automated colorimetric assay developed by Boutin and Brodeurr(1970) for the determination of human serum pseuodocholinesterase variants.

6. Ability to alter and control variables.

The determination of cholinesterase may be influenced by variables including temperature, pH, ionic strength of the reaction medium and the concentration of the reagents. Hence it is important that in a chosen method such variables should be readily controlled and, if necessary, altered. Sometimes this may present difficulties. For example in the Warburg manometric technique the normal working pH of 7.4 can be varied only over a relatively small range (Nachmansohn and Wilson, 1955; Augustinsson, 1957; Witter, 1963). The problems of controlling, or correcting for, temperature, the instability of reagents and standards and the problems of assessing colour changes resulting from reactions in the field were discussed by Witter (1963).

General considerations concerning methods for detecting and determining cholinesterase.

It has been stated (Witter,1963) that there are two prerequisites for a satisfactory assay proceedure for cholinesterases:-

1. The rate of reaction measured must be proportional to the amount of enzyme present.

2. The enzyme measured under the conditions of assay must be a cholinesterase.

Meeting the first of these prerequisites depends upon an appropriate choice of enzyme and substrate concentrations, preferably the measurement of reagent appearance or disappearance over several time intervals and control of the pH, the ionic strength of the reaction medium and the temperature (Witter,1963).

Establishing the identity of the enzyme under assay may depend upon the use of selective, specific, substrates and inhibitors (Augustinsson, 1949; 1957; 1963; 1971). The availability of purified and

stable cholinesterase preparations facilitates assay proceedures in which a standard of cholinesterase activity is required (Augustinsson, 1957). Furthermore purified enzyme preparations may be free of endogenous substances, such as salts, which may be present in tissue homogenates and which may influence cholinesterase activity (Augustinsson, 1963). Methods of purifying cholinesterases have been reviewed by Augustinsson (1957; 1963) and Usdin (1970). Here, as with the methods of assay themselves, the application of new experimental techniques may prove useful. Recently affinity chromatography has been employed for the purification of acetylcholinesterase from electric eel (Ashani and Wilson, 1972; Rosenberry, Chang and Chen,1972) and from both electric eel and bovine erythrocytes (Berman and Young,1971).

Other factors may have an important bearing on the results of a cholinesterase determination. For example Pavlic (1967) showed that TRIS buffer solution could inhibit cholinesterases and warned that this should be taken into account when appropriate. Ellin (1972) and Ellin, Groff and Kaminskis (1972) reported on the introduction of an error into the measurement of blood cholinesterase consequent upon the use of a secondary reference standard, glutathione, in preference to thiocholine, a primary standard, in the use of the colorimetric method of Ellman. Witter, (1963) pointed out the potential inhibition of cholinesterases by ionic detergents which may be used to clean glassware involved in enzyme determinations. These examples should serve to show that all aspects of the method chosen for cholinesterase determination should be given careful consideration.

Choice of methods for the present research.

It was decided to use both the Warburg manometric technique and the automated pH - stat technique for acetylcholinesterase determinations in the present research. The former method was chosen because of its potential accuracy and reliability and the latter method for its potentially high precision and its convenience (Augustinsson,1971). Additionally the choice of methods was influenced by the existing availability of the equipment required in the use of the two techniques. The facility with which alterations could be made to the pH at which the acetylcholinesterase determinations were performed and the facility for performing kinetic studies were further factors leading to the selection of the pH - stat method, since it was envisaged that the present research would give rise to a need to study acetylcholinesterase activity in the presence of enzyme inhibitors at different pH values.

The Warburg manometric method for the determination of cholinesterase activity.

This method estimates the acid produced by cholinesterase-catalysed hydrolysis of ester substrates by causing the acid to liberate carbon dioxide ($C\theta_2$) from a bicarbonate ($NaHCO_3$)- carbonic acid buffer in which the reaction takes place. Thus the measure of liberated CO_2 is equivalent to the production of acid which in turn relates to enzyme activity under the given conditions. The reaction takes place in a closed vessel attached to a manometer, the volume of CO_2 produced being estimated from the change in pressure, at constant gas volume and constant temperature, over a period of time (often thirty minutes). Passage of CO_2, in nitrogen, through the reaction vessel during the experiment serves to fix the concentration of carbonic acid. As with the titrimetric methods described (see pages 40 et se correction of results, produced by manometric methods, should be made in respect of acid liberated by non-enzyme-catalysed mechanisms. Additionally corrections should be made for thermobarometric changes during the experiment. The reactions may be run in the presence of enzyme inhibitors.

Manometric techniques have been applied to the estimation of exchange of gases in both chemical and biological reactions. Warburg (1926) reviewed the history of the observation of gas reactions by manometric methods. In the case of biological reactions thermanometric instruments, often termed respirometers, have been used for measurement of the oxygen uptake or CO_2 production associated with cell and tissue respiration. (Warburg,1926; Dixon, 1952; Umbreit, Burris and Stauffer, 1964). The principles of manometry, types of manometer, variations in experimental technique and some applications of manometric methods have been reviewed by Dixon (1952) and Umbreit et al (1964).

Essentially the Warburg manometric technique utilises a reaction flask with one or more side arms and often a centre well in which can

be placed alkali for CO_2 absorption in those experiments requiring measurement of oxygen uptake uncomplicated by CO_2 production. The flask is connected to a manometer containing a liquid of known density and temperature control is achieved by immersing the reaction flask in a thermostatically controlled water bath. The manometer is fixed outside the water bath so that readings of pressure changes can be taken during the course of the reaction after adjusting the fluid in the manometer, prior to each reading, to ensure constant gas volume in the reaction system. To facilitate gas production and uptake the flask is shaken in the water bath between readings.

In the case of cholinesterase determinations the reaction medium is placed in the main part of the flask whilst the enzyme preparation, substrate solution or inhibitor solution may be placed either in the main part of the flask or in the side arm depending on the requirements of the experiment (Augustinsson, 1948;1957;1971). At the appropriate time the reaction flasks are tilted to mix the previously seperated enzyme and substrate solutions to start the reaction; readings of CO_2 production, with time, are then taken.

Other types of manometric technique have been applied in studies of cholinesterases. A well known example was the application of the Barcroft differential manometer (see Dixon,1952; Umbreit et al,1964) by Stedman and Stedman (1935) to the determination of the relative cholinesterase activities in blood cells and serum.

The first application of the Warburg technique to cholinesterase determinations was made by Ammon(1933). He investigated various tissues for cholinesterase activities and showed that certain compounds, notably physostigmine and prostigmine, already shown by biological methods to inhibit the enzymatic hydrolysis of AcCh, exhibited inhibition towards cholinesterase under the condition of the test.

Since then detailed descriptions of the use of Warburg manometry in cholinesterase studies have been given by Augustinsson (1948;1957),

Mendel and Hawkins (1950), Witter (1962) and Bockendahl and Ammon (1965).

The review of Augustinsson (1957) showed that, as with other methods of cholinesterase determination, the Warburg method has been used with many variations in the experimental conditions. It was also shown by Smallman and Wolfe (1954) that the presence of $NaHCO_3$ in the Warburg method reduced both the absolute activity of the cholinesterase and its relative activation by other salts which may be present in the reaction medium. Sodium chloride should be added to the reaction medium to overcome the effect of $NaHCO_3$ on the enzyme (Witter,1963). These factors can render a comparison of results from different sources potentially difficult.

Usdin (1970) claimed that the major disadvantages of the method were the inability to control pH during the reaction; the ability to run only a few samples at a time; and its inapplicability when rates must be determined within a short time of enzyme-substrate mixing. However, the automated pH - stat method can only handle one determination at a time on any one set of apparatus whilst Warburg instruments can handle several samples at a time (see pages 48 et seq) Hardegg and Schaefer (1952) by designing apparatus which allowed enzyme-substrate mixing with the reaction vessel remaining in the water bath were able to avoid disturbing the temperature equilibrium of the system. This allowed immediate recording of reaction velocities. Further Baron, Casterline and Orzel (1966) were able to abbreviate the Warburg technique by taking readings as often as every fifteen seconds for as short a time as two-and-a-half minutes. This was in connection with the study of carbamate inhibitors of cholinesterase which rapidly dissociate from the enzyme in the presence of certain substrates.

Witter (1963) and Augustinsson (1971) stated that the rate of hydrolysis of substrates cannot be studied by the Warburg method at concentrations below about 0.4 m mol/l because insufficient gas is liberated. Myers (1952) has shown that the ability to detect gas

production, and thus to measure hydrolysis rates, in the presence of low substrate concentrations, is governed not only by the concentration of substrate but also by the total amount of substrate present and the frequency of manometer readings. If necessary use can be made of extrapolations when it is desired to determine the gas volume which would have been produced under these circumstances in longer periods of time (e.g. in thirty minutes).

A point of contrast between the Warburg and pH - stat methods may be seen by comparing the reviews of Augustinsson (1957;1971). Between these dates the general Warburg method had not been improved in any important detail and the automatic recording possibilities mentioned in the first review had not received further study (Augustinsson, 1971). By contrast the method of continuous titration had been automated and the resulting system well utilised during this period.

The Warburg manometric method has, nevertheless, found widespread application in a number of areas of cholinesterase research. Human blood cholinesterase activity, for example, has been studied by Augustinsson (1955b)whilst Augustinsson and Holmstedt (1965) used the method on blood samples originating in field tests. Witter (1962) made a study of the conditions necessary for the quantitative manometric assay of blood cholinesterases in the rat and in 1963 referred to the use of the method in the analysis of autopsy specimens for cholinesterase activity. Assessments of cholinesterase inhibitors have employed the Warburg method (for example Baron et al, 1966; Dubois, Kinoshita and Frawley,1968).

This method has also had applications as diverse as studies of the relationships between erythropoiesis and cholinesterase erythrocyte levels (Bhatnager, 1968) and in studies concerning the stability of serum cholinesterase (Beckett, Vaughan and Mitchard. 1969).

The pH - stat method for the determination of cholinesterase activity.

This method involves continuous titration of acid, liberated by the enzyme-catalysed hydrolysis of an ester substrate, with standard alkali in a suitable reaction vessel at a known and constant pH and over a given period of time. Correction of the result is made, if necessary, for acid released by other mechanisms such as by the spontaneous, or non-enzymatic, hydrolysis of the substrate. Measurements may also be made where the cholinesterase-catalysed reaction takes place in the presence of enzymeinhibitors. The amount of alkali used is related to the production of acid and hence to both the hydrolysis of the substrate and the activity of the enzyme under the given experimental conditions. An ability to measure, conveniently, enzyme-catalysed hydrolysis as a function of time makes the method very suitable for kinetic studies involving these reactions.

Renshaw and Bacon (1926) measured the rates of non-enzymatic hydrolysis of certain choline esters at pH7.8 and 37°C by determining the rate at which dilute alkali ($Ba(OH)_2$) needed to be added to keep the reaction mixture at pH 7.8 as judged by the use of cresol-red as indicator in both the reaction mixture and in a colour standard. Stedman, Stedman and Easson (1932) utilised this technique of continuous titration for the determination of choline ester hydrolysis catalysed by an enzyme in horse blood serum for which they proposed the name choline-esterase. This experiment involved control of the reaction temperature (30°C) and the recording every five minutes, for a period of twenty minutes, of the burette readings as alkali was run in dropwise to maintain pH at the predetermined end point. Amongst points noted were the buffering action of the serum proteins; the fact that the colour of the serum could modify the colour of the indicator and that at pH 7.4 spontaneous hydrolysis with both acetylcholine and butyrylcholine was almost negligible. In a subsequent paper (Stedman,

Stedman and White,1933), in which the activity of blood serum cholinesterase was determined in various species, an early attempt is seen to measure enzyme activity in small samples (1 ml.) of blood.

Hall and Lucas (1937) employed an indicator method of continuous titration to investigate whether there was any correlation between various physiological parameters and serum cholinesterase levels in both normal humans and those suffering from a variety of pathological conditions. This study involved the use of an automatic microburette in the titration.

Glick (1937) introduced the use of the glass electrode to the titrimetric measurement of cholinesterase-catalysed hydrolysis thus obviating the need to use indicators. This represented an improvement in technique as different indicators and buffers would be needed when the pH of an end point required to be altered; such indicators and buffers might affect enzyme activity. Further the use of the glass electrode sought to allow study of enzyme-substrate reactions without the introduction of a foreign substance into the reaction mixture. The reaction rate was measured by recording the number of drops of titrant (0.02 or 0.01 N NaOH) added in each five minute period for a total of thirty minutes. A linear relationship was shown between enzyme concentration and activity at pH 8.4 whilst activity-pH relationships were studied over the pH range 5 to 10. Scoz and Cattaneo (1937) also used an electrometric technique but employed a quinhydrone electrode instead of a glass one.

It was claimed by Alles and Hawes (1940) that, of the chemical methods then available for cholinesterase determination, continuous titration with a glass electrode was the technique of the greatest precision, simplicity and versatility with the least danger of interference from added reagents or manipulation. Readings were made of the times required to use measured amounts of alkali during the course of the reaction and the reaction rates calculated as the volumes

of 0.02N NaOH used in twenty minutes with due correction for alkaline hydrolysis. Above pH 8.0 it was considered that the accuracy of the titrations decreased rapidly because the correction for alkaline hydrolysis became a large fraction of total hydrolysis and because the unavoidable slight fluctuations of pH about the selected end point have a more marked effect on alkaline than on enzymatic hydrolysis.

Yet a further variation of titrimetric technique was the use by Delaunois and Casier (1946;1948) of an antimony electrode rather than a glass one.

Brown and Bush (1950) found electrometric titration of value in the monitoring of blood cholinesterase levels in workers exposed to the insecticide parathion, an organophosphorus cholinesterase inhibitor. Kaufman (1954) utilised electrometric titration in a study designed to evaluate serum cholinesterase activity as a test of liver function. Chouteau, Rancien and Karamanian (1956) calculated reaction rates, determined electrometrically in the presence of blood serum cholinesterase, directly from the slopes of straight-line graphs showing the rate of hydrolysis of substrate.

The micromodification of such electrometric titration methods may be illustrated by the paper of Radouco and Frommel (1952). Here a small reaction vessel was described incorporating a combined electrode, a magnetic stirer and a microburette, thus permitting a reduction in the volumes of reagents used.

An important advance in the development of electrometric titration techniques came with the design of automatic recording instruments thus increasing the potential usefulness of these techniques. Nielands and Cannon (1955) described apparatus for the automatic determination of the volume of titrant added as a function of time at a constant pH. The usefulness of their apparatus in enzyme research was illustrated by experiments with acetylesterase from orange peel and with lactic dehydrogenase. It was thus shown that the use of the apparatus was not

restricted to hydrolytic enzymes and that it was suitable for use with rapid reactions.

Both Jensen-Holm, Lausen, Milthers and Møller (1959) and Jørgensen (1959) described the use of a commercially available automatic recording titrator (Radiometer; Copenhagen). Such instruments incorporate a burette which automatically delivers titrant in response to fluctuations of pH from the preset end point as detected by the pH electrodes in the reaction medium. In the case of reactions producing acid it is the fall in pH below the end point which activates the burette to add alkali. Addition of the titrant is shown either on a digital display, a chart recorder, or on both. Jensen-Holm et al (1959) employed the instrument to determine the cholinesterase activity in blood and in a variety of animal tissues. Before the substrate was added the enzyme preparation was titrated for some minutes to check for the non-specific liberation of acid by the tissue preparation. After substrate addition, titration of the enzyme-catalysed reaction then proceeded for about four minutes or more. It was shown that whilst non-specific acid liberation was rare with fresh blood it could be significant with other tissues, possibly influenced by the storage of the tissues post-mortem,. This could lead to serious error in cholinesterase determinations and must be corrected for where appropriate. It was recognised that the initial part of the titration curve might be unduly steep when a substrate solution having an acid pH is added to the reaction medium. Such a substrate solution might reduce the pH of the reaction medium below the pre-set end point and cause addition of alkaline titrant to restore the pH before the enzyme-catalysed reaction can be recorded under the predetermined conditions.

Automatic recording titrators have become recognised tools for the titrimetric monitoring of reactions involving cholinesterases. Delaunois (1962) and Nabb and Whitfield (1967) have reported on the use of such instruments. Nabb and Whitfield (1967) applied their instrument

to gain a well defined and precise measurement of the effects of organophosphorus insecticides upon cholinesterases. They claimed that the automated procedure should be capable of application for cholinesterase determinations in most situations.

The use of the term 'pH - stat' to describe methods involving continuous titration to a fixed end point with automatic recording titrators has become accepted (Nabb and Whitfield,1967; Serat and Mengle,1973 for example).

At the present time automatic titration of acid released by cholinesterase catalysed hydrolysis is generally performed in an atmosphere of nitrogen. Ballantyne (1968b) has shown that uptake, by the reaction media in electrometric titration, of acidic substances from the air can occur causing alkaline titration and leading to false estimates of enzyme activity. The existence of air pollution with its regional and temporal variations can add to the problem. It was shown that titration in an inert atmosphere of nitrogen overcame the problem and it is now usual to pass nitrogen through the reaction vessel close to the surface of the reaction medium.

Many of the variations in non-automatic indicator and electrometric methods of titration involving cholinesterases have been listed in the review of Augustinsson (1957). Similarly some variations of the titrimetric method employing an automatic recording titrator have also been reviewed (Augustinsson 1971). Such variations include differences in the volume of the reaction medium; pH of the end point of the titration; ionic composition of the reaction medium; source and type of cholinesterase; substrate; reaction time; and normality of the titrant (NaOH). Hence comparison between results of cholinesterase determinations derived by the use of titrimetric techniques may be difficult.

Serat and Mengle (1973) recognised that one of the most serious considerations in assessing damage to persons exposed to pesticides was

the confidence which could be placed on laboratory determinations of cholinesterase activity. Problems encountered included the involvement of many laboratories of varying degrees of competance in such determinations and a frequent lack of knowledge concerning the sensitivity of the assay methods used and the exercise of quality control. They conducted a survey of interlaboratory variation in the cholinesterase activities determined in blood samples using the pH -stat technique of Nabb and Whitfield (1967).

Problems in the interpretation of results arising from the use of different combinations of variables and from an attempt to compare results from different laboratories may therefore be met in the use of the pH - stat method.

EXPERIMENTAL WORK.

EXPERIMENTS USING THE WARBURG

MANOMETRIC METHOD TO DETERMINE

ACETYLCHOLINESTERASE (AcChE) ACTIVITY.

An investigation into the use of the Warburg manometric method for the determination of acetylcholinesterase actvity.

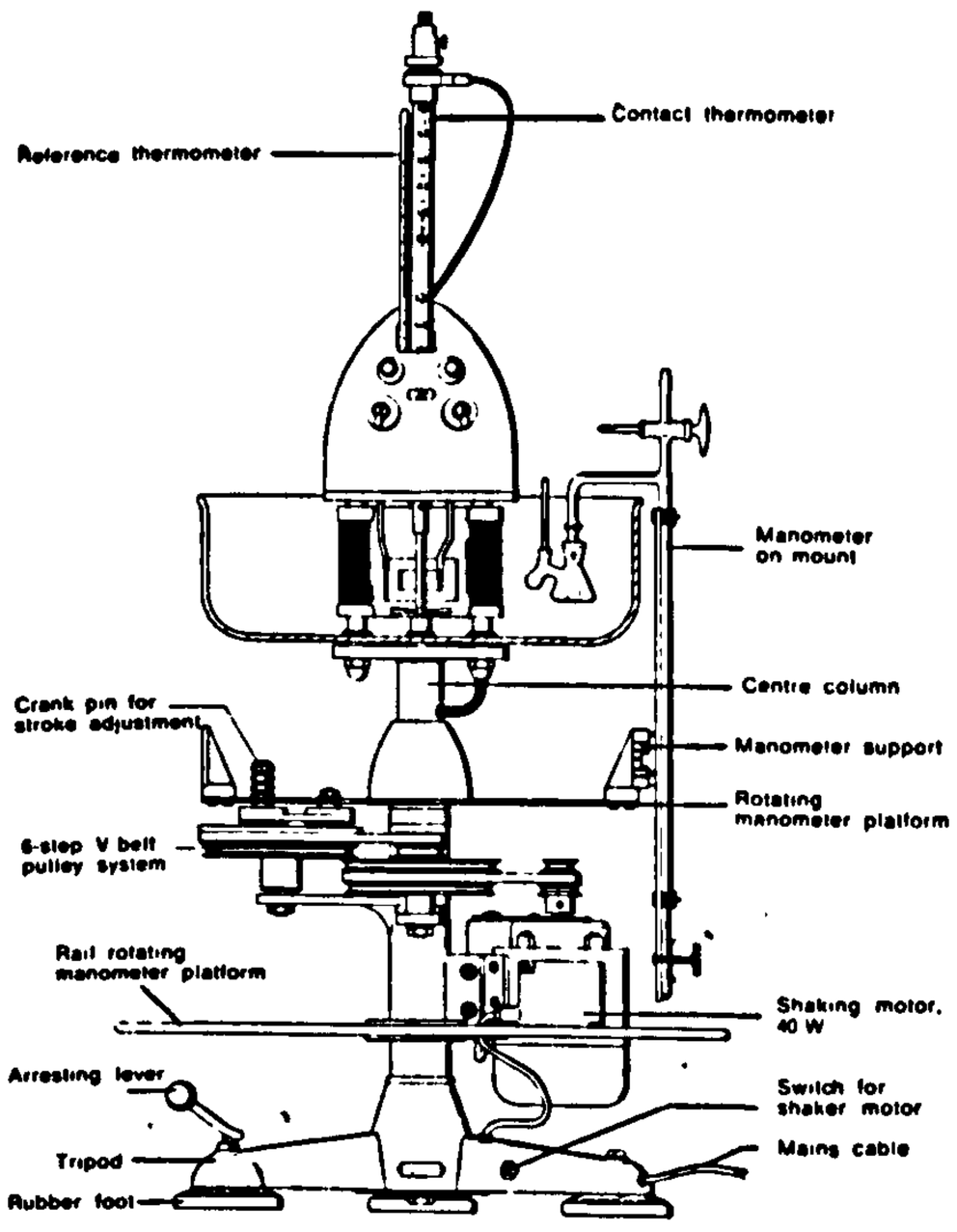
It was decided to investigate the use of the Warburg manometric method for the determination of acetylcholinesterase activity by applying the method to a study of the interaction between AcChE and AcCh.

Principle of the method

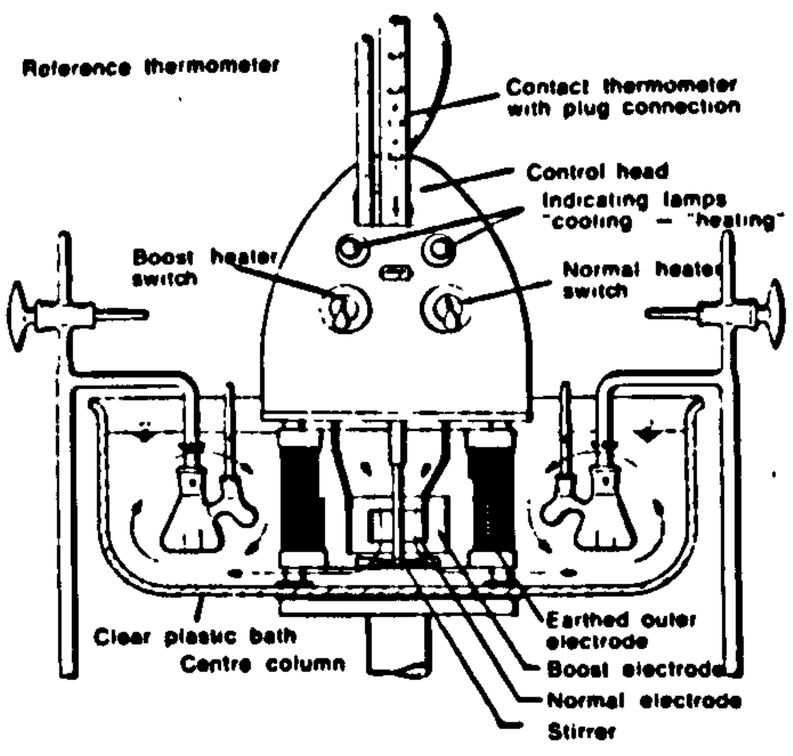
This method as described in the Introduction (p36-39) depends upon the measurement of CO_2 liberated from an aqueous bicarbonate-carbonic acid buffer medium consequent upon the reaction of the bicarbonate with acid produced by the enzyme-catalysed hydrolysis of an ester substrate (here a choline ester). The CO_2 liberated is measured as a pressure change at constant volume. The pressure change is then converted to a volume change (μl CO_2) by the use of a flask constant which relates to the volume of the particular reaction vessel. In turn this CO_2 volume change is related to the time elapsed since the start of the reaction. A frequently used expression of activity for an enzyme reaction system is the μ l CO_2 produced in a reaction time of 30 minutes. Several such reaction systems will be utilised to determine the activity and kinetic parameters of an enzyme-substrate or an enzyme-inhibitor reaction.

Apparatus.

The apparatus (Fig 3) used in the present study was the Warburg apparatus (Braun Type V85) having a circular water bath capable of supporting up to 14 manometer-reaction flask systems. The conical reaction flasks were of approximately 13-15 ml volume and were supported within the water bath with their manometers on its outside. One limb of each manometer was open to the atmosphere. Each reaction flask had a side arm, which allowed the enzyme and substrate to be kept separate until it was desired to mix them in order to start the reaction. All manometer and flask joints were greased with yellow soft paraffin.



Arrangement of shaking system



Schematic section through water bath

Fig 3.

Diagram of Warburg manometric apparatus.

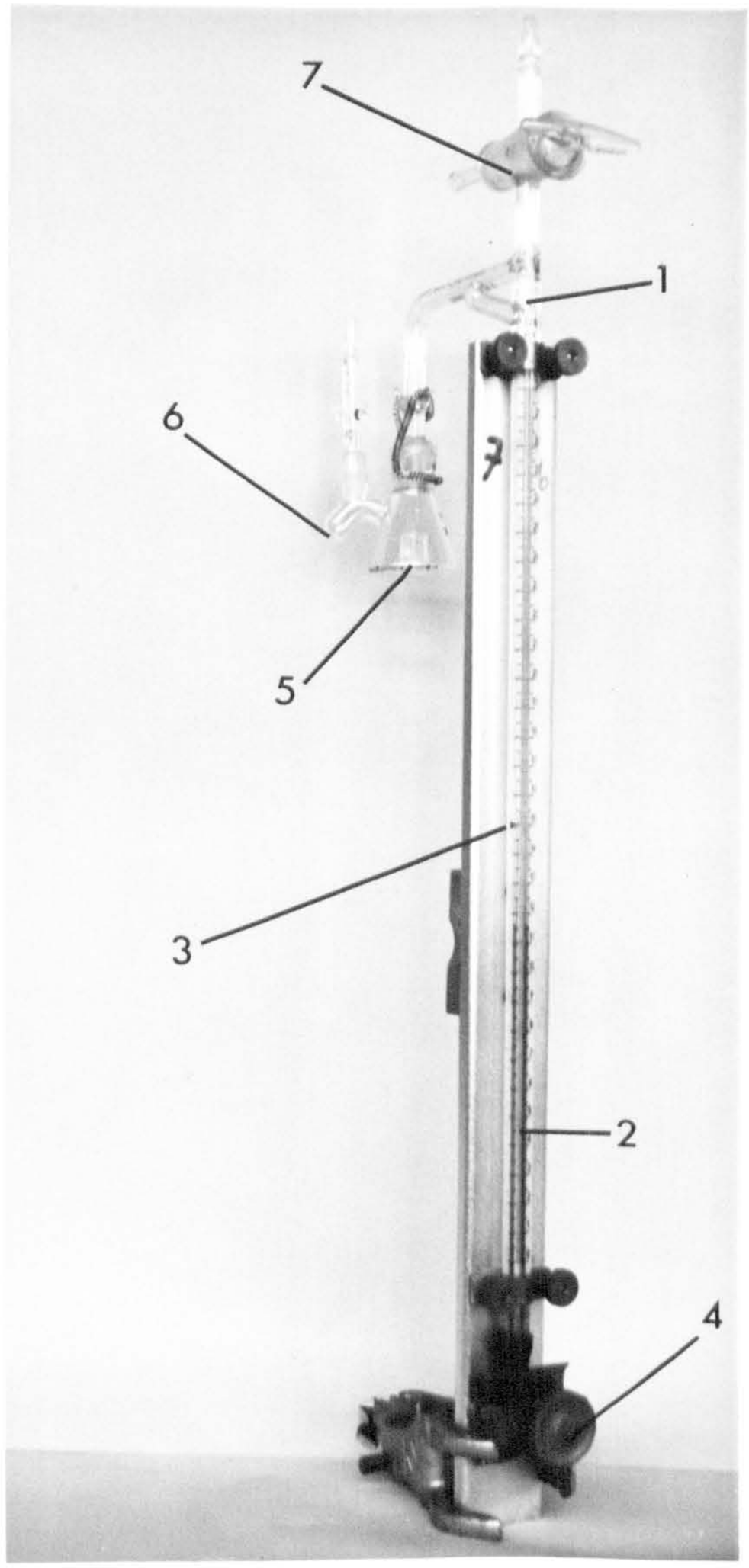


Fig 4.

Manometer and reaction flask.

1. Manometer.
2. Brodies Solution.
3. Reference point for readings.
4. Screw for adjusting level of Brodie's Solution.
5. Reaction flask.
6. Side arm of reaction flask.
7. Manometer stopcock.

Each flask was assigned to a manometer (Fig 4) which contained Brodie's manometer solution having a density of 1.033g/cm^3, such that 10,000 mm Brodie's solution corresponded to 760 mm. Hg. The volume of each manometer from the junction with its reaction flask to a reference mark (the mid-point) on the manometer was known. The reference mark was on the manometer limb nearest to the reaction flask and indicated both the initial level of the Brodie's solution and also the level to which the Brodie's solution had to be readjusted from a reservoir prior to a pressure reading during the course of the reaction. The readjustment ensured that the pressure changes were read at constant volume.

The volume of a reaction flask and its manometer, as described above, together constituted the 'total' volume for that flask and manometer. The total volume of the solutions placed in any *one* reaction flask comprised the fluid volume (Vf) in the flask. The difference between the total volume and Vf constituted the 'gas volume' (Vg) for that particular manometer/flask system. These volumes were used in the calculation of the flask constant.

Each manometer had a stopcock and each reaction flask a gas vent, such that both the portion of manometer from the flask to the start of the Brodie's fluid and also the reaction flask could be open to the atmosphere, and could be saturated with 5% CO_2/95% N_2 to fix the concentration of carbonic acid in the reaction medium.

Control of the temperature of the water bath was by a heater working in conjunction with a contact thermometer preset at the required temperature of the experiment. Heating of the bath was by conduction, electrical current being passed between two concentric heating electrodes and an earth electrode through a conduction medium comprising distilled water containing a prescribed amount of sodium chloride. The speed of heating was governed by the use of one, or both, heating electrodes ('normal' and 'boost', Fig 3) and the concentration of

the salt solution. Achievement of an even temperature throughout the water bath was aided by the incorporation of an electrically driven stirrer within the bath. The variation in the bath temperature was within the limits $< \pm$ 0.01°C. Cooling and heating periods in the temperature control cycle were indicated by lamps on the apparatus.

The shaking mechanism of the apparatus was adjusted to give 80 oscillations per minute with a travel of approximately 5 cm. per oscillation. It was possible to rotate the apparatus so that any single manometer was in front of the operator. This facilitated the reading of the manometers.

Solutions.

Reaction Medium (B)

This medium had the following composition:-

NaCl	0.15 mol/l
$MgCl_2$	0.04 mol/l
$NaHCO_3$	0.034 mol/l

The salts which were kept as stock solutions were of Analar grade (B.D.H) the medium being prepared in deionised water. The composition of (B) was such that when the solution was saturated with 5% CO_2/95% N_2 at 37°C it had a pH of 7.4.

The gas mixture, 5% CO_2/95% N_2, was obtained commercially from the British Oxygen Co. Ltd.,

(B) was prepared in batches of 1 litre and stored in the dark at 4°C between experiments. It was 'regassed' with 5% CO_2/95% N_2 prior to each experiment.

Enzyme Solutions (E)

The enzyme used in the investigations was AcChE from bovine erythrocytes, obtained commercially as a lyophilized powder in 50 unit quantities, from Sigma Chemical Co. One unit of AcChE would hydrolyse 1 μ mole AcCh to choline and acetate per minute at pH 8.0 and 37°C.

This preparation, as received, contained gelatin, NaCl and

sodium phosphate buffer. A master solution of enzyme was prepared containing 1.25 units/ml by dissolving 50 units in 40 ml. of (B) containing 1% partially hydrolysed gelatin made by digesting gelatin in deionised water on a steam bath for 8 to 10 hours as described by Kay (1968). The purpose of the gelatin was to stabilise the master enzyme solution which was stored in the refrigerator, in the dark, at $4^{o}C$ between experiments. Prior to each experiment a fresh dilution of the master enzyme solution was made in (B) 'gassed' with 5% CO_2/95% N_2 to give an enzyme solution of the concentration required for the experiment.

Substrate solutions (S)

The substrate used in the studies was AcCh perchlorate (B.D.H. Biochemical Grade). This salt was used in contrast to other salts, particularly the chloride, because the perchlorate of AcCh is non-hygroscopic (Carr and Bell 1947). For each experiment, a fresh master solution of substrate was prepared in deionised water. This was diluted in 'gassed' (B) to give the concentrations [S] required by the experiment.

Temperature.

The temperature at which the experiments were conducted was $37^{o}C \pm 0.01^{o}C$.

Reaction systems for enzyme-substrate studies.

In order to measure the enzyme-catalysed hydrolysis of substrate at a given enzyme concentration [E] and at a given substrate concentration [S] four reaction systems were thought to be necessary for accuracy.

These were:-

1. A thermobarometer (TB) to compensate for effects of fluctuations in temperature and atmospheric pressure on the gas measurement.

2. An enzyme-buffer blank (EB) to allow for any evolution of CO_2

caused by the enzyme preparation.

3. A blank (SB) containing substrate and reaction medium . to measure spontaneous hydrolysis of substrate.

4. A flask containing enzyme, substrate and reaction medium (ESB). From four such reaction systems was derived a compensated reaction rate under stated conditions.

An experiment involving more than one concentration of substrate ($[S_1]$, $[S_2]$, $[S_3]$ etc.) but a single concentration of enzyme $[E]$ was able to utilise one TB flask and one EB flask, with pairs of flasks (S_1B_1, ES_1B, S_2B, ES_2B etc.) to compensate for spontaneous hydrolysis at the different concentrations $[S_1]$, $[S_2]$ etc.

The flasks ESB contained 0.5ml. (E) 1.0 ml.(S) 1.0 ml.(B)

The flasks SB contained 1.0 ml.(S) 1.5 ml.(B)

The flask EB contained 0.5 ml. (E) 2.0 ml.(B)

The flask TB contained 2.5 ml. deionised water.

Thus Vf was always 2.5 ml

Experimental protocol for enzyme-substrate studies.

The reaction flasks were loaded according to their designated use as outlined above, the required volume of (E) being pipetted into the side arm where appropriate, and the required volume of other reagents being pipetted into the main body of the flask. Care was taken to avoid getting reagents in the centre well of the reaction flasks because use of this well was not required for the experiments and reagents trapped therein might have been effectively removed from the reaction under investigation.

Each reaction flask was attached to its manometer, the volume of both flask and manometer being recorded to give the 'total' volume of the reaction system.

With the manometer stopcock and reaction vessel gas vent open to atmosphere, each reaction flask was placed in the water bath with its manometer supported on the outside of the water bath. A fifteen

minute period of equilibration ensued during which the reaction flask assumed the temperature of the water bath. During this time 5% CO_2/ 95% N_2 was passed through the open stopcock of the manometer, through the attached reaction flask, and out to atmosphere through the gas vent. This 'gassing' of the reaction flasks brought the pH of the medium to its predetermined value, as governed by the concentration of $NaHCO_3$ and the temperature of the experiment. The process of equilibration for temperature and pH was facilitated by shaking the reaction flasks in the water bath during the period of 'gassing'.

After temperature and pH equilibration, the level of the Brodie's solution in each manometer was adjusted by a screw control to the reference mark and any difference in the heights of the limbs was noted, so that subsequent pressure readings could be corrected for this discrepancy.

The stopcocks of the manometers and the gas vents of the reaction flasks were closed and the side arm enzyme contents of each flask mixed, where appropriate, with the contents of the main compartment of each flask.

In order to mix the contents of the reaction flasks the flasks and their manometers were removed from the water bath and the mixing effected by a tilting movement. Throughout this period the end of the limb of the manometer open to the atmosphere was covered by a finger. This prevented the Brodie's solution being sucked from the manometer into the reaction flask due to the temperature change in the flask consequent upon its removal from the water bath. Again care was taken not to get solutions into the centre well of the flasks.

This was the start of the reaction, the shaking mechanism of the apparatus being switched on, and the time noted. The shaking now served to facilitate the enzyme-substrate reaction and the resultant evolution of CO_2 from the reaction medium.

Reading of manometers.

At given times (e.g. 5,10,15 minutes etc.) after the start of the reaction the shaking was stopped, the manometer fluid was adjusted to the reference mark and the difference (h.mm), if any, in the heights of the limbs of the manometers was noted. This reading was corrected for any initial discrepancy in the height of fluid in the two limbs.

The pressure of gas evolved by any reaction system at any time was converted to give the volume of CO_2 evolved at that time. For any given manometer/flask system this conversion involved multiplying the h(mm.)reading at a stated time by the 'flask constant' (k) for that manometer/flask system.

Thus h(mm.) x k = Vol (μ1) of CO_2

Flask constants.

The flask constant takes into account the factors which depend on the reaction system (manometer and flask volumes) and the particular experimental conditions.

For any manometer/flask combination:-

$$k = \frac{V_g \frac{273}{T} + Vf\,\alpha}{Po}$$

when Vg is the 'gas volume' (μ1)

Vf is the 'fluid volume' (μ1)

Po is standard pressure (10,000 mm Brodie's fluid)

T is the temperature at which the experiment is conducted (^{o}K)

α is the gas solubility in the reaction medium under stated experimental conditions. It is expressed as μ1 gas/μl liquid and its value, which depends on the gas, here CO_2, the liquid, and the experimental condition is derived from tables (Dixon,1952;Umbreit et al,1964).

For convenience the flask constants were, prior to the experiments, calculated for 26 combinations of manometers and flasks for a

temperature of 37°C and for values of Vf equal to 2500 µl (for enzyme-substrate studies) and 3500 µl (for possible enzyme-substrate-inhibitor studies).

Calculation of results.

For each of the flasks ESB, EB and SB the measured volume change had to be corrected for the volume changes, if any, in the thermobarometer.

Thus Vol.ESB - Vol.TB represented the total volume change due to enzyme catalysed hydrolysis of the substrate.

Vol. EB - Vol.TB represented the correction for any CO_2 evolution due to the interaction between E and B.

Vol.SB - Vol.TB represented the further correction for any spontaneous hydrolysis of the substrate.

The corrected volume change (y, µlCO_2) at any given time, for interaction between the stated concentrations [E] and [S] was given by:-

$$y = \text{Vol. (ESB - TB)} - \text{Vol. (EB -TB)} - \text{Vol. (SB - TB)}$$

$$\text{Thus } y = \text{Vol. ESB} - \text{Vol. EB} - \text{Vol. SB} + \text{Vol.TB}$$

For a given experiment a graph of y against time was plotted. The resultant points were examined to ascertain the nature of the relationship between these variables. Where this was a straight line (most cases) a regression analysis was performed and the b_{30} value (Augustinsson, 1948) derived, by extrapolation if necessary.

The b_{30} value was the chosen expression for reaction velocity and was the number of µl of CO_2 produced in 30 min. after correction of the results as described.

It should be understood that the b_{30} value was a measure of the reactiom velocity resulting from a given concentration of substrate reacting with a given concentration of enzyme. under stated experimental conditions. By varying the concentration [S] at fixed enzyme concentration, and under fixed experimental conditions, a series

of b_{30} values could be obtained and from this the kinetic parameters K_M and V_{MAX} derived.

An example of the calculation of b_{30} values, K_M and V_{MAX} is given in the Appendix(p. 118-123).

Initial experiments involving the Warburg manometric technique for the measurement of AcChE activity.

The effect of the relative concentrations of enzyme [E] and substrate [S] on the measurement of reaction velocity for the AcChE catalysed hydrolysis of AcCh was investigated. Information was also sought concerning both the frequency of the manometric readings required during an experiment and the total duration of the experiment necessary to achieve reproducible reaction velocity measurements at stated concentrations [E] and [S] .

Six experiments were performed in which the final concentration of substrate in the reaction flask, F.F.C [S], varied within the range 0.125 to 4.00 m mol/l in each experiment and the final flask concentration of enzyme, F.F.C [E] , was either 0.05 or 0.10 units/ml. A single batch of enzyme was used for the six experiments. In each experiment manometric readings were, in general, made every ten minutes, the total duration of the experiment being forty minutes. The results are shown in Tables 4 and 5.

A further five experiments were performed with a fresh batch of enzyme in which the concentration [E] was equal to 0.025 units/ml and [S] varied within the range 0.25 to 8.0 m mol/l in each experiment. It was considered that here, where the concentration [E] was lower relative to [S] compared with the previous experiments, the initial linear relationship between CO_2 production and time would persist for a longer period as the substrate would not be used up so quickly. Thus the regression analyses involved in the derivation of the b_{30} values should have been more accurate. To this end the period for which each experiment was run was lengthened from forty to ninety minutes but a time interval of ten minutes was still generally retained between readings. These results are summarised in Tables 6 and 7.

Results and Discussion.

Table 4 shows the b_{30} values (Augustinsson, 1948) from the six

experiments derived for the reactions between stated concentrations [S] and [E].

For a given combination of [S] and [E] in each experiment the b_{30} value was derived by the application of regression analysis to those corrected calculated CO_2 volumes (y µl) which, by inspection, bore a rectilinear relationship to time. Derivation of b_{30} values involved, also, correction for the corresponding extrapolated values of y at zero time (see Appendix). In some instances (marked *in Table 4) regression analyses were not applied as the values of y appeared to be linearly related to time only over two readings. Here an estimate of b_{30} was made by extrapolating a line drawn through the two points and correcting for the value of y at zero time.

Table 4 showed that in many instances the linear relationship between y and time did not obtain for the whole experiment. This represented the situation where [S] was low relative to [E] and where most of the substrate had probably been hydrolysed before the end of the experiment (Myers 1952). When [E] was 0.05 units/ml. the initial linear relationship between y and time had ceased before the end of the experiment at values of [S] equal to 1.0 m mol/l or less. When [E] was raised to 0.10 units/ml. the same phenomenon was observed at values of [S] equal to 2.0 m mol/l or less. Thus where [E] was increased relative to [S] the period of the initial linear relationship between y and time shortened.

It is seen from Table 4 that the errors of the slopes of the regression lines, y on time, could vary widely for a given combination of [E] and [S] and could be very large. The large errors possibly reflected the small numbers of values involved in the individual regression analyses as manometric measurements were only made at ten minute intervals for a maximum of forty minutes.

In Table 5 the mean b_{30} values derived from the results are given. There was a tendency for the mean b_{30} value to increase as [S] increased

Table 4

Results of manometric measuremnts of reaction velocity for the AcChE (E) catalysed hydrolysis of AcCh (S) at 37°C.

Expt.	[E] units/ml	[S] m mol/l	y. linearly related to time for period (mins)	Slope (b) of line y. on time	Number of points on line	Error of slope $\frac{(Sb)}{b}$ x100%	b_{30} value $\mu l CO_2$/30 min
3	0.05	4,00	0 - 40	1.9803	4	11.50	59.41
4	0.05	4.00	0 - 40	1.5205	3	2.94	45.61
1	0.05	2.00	0 - 40	1.6982	4	7.83	50.91
2	0.05	2.00	0 - 40	1.7114	4	8.24	51.35
3	0.05	2.00	0 - 40	2.0198	4	12.21	60.60
4	0.05	2.00	0 - 40	1.5522	4	9.79	46.57
1	0.05	1.00	0 - 20	*	2	—	35.50+
2	0.05	1.00	0 - 30	0.6606	3	6.42	19.81
3	0.05	1.00	0 - 30	1.7885	3	6.15	53.66
4	0.05	1.00	0 - 40	0.6510	3	10.63	19.53
1	0.05	0.50	0 - 20	*	2	—	51.50+
3	0.05	0.50	0 - 40	0.2030	4	113.60	6.09
4	0.05	0.50	0 - 30	0.8260	3	38.45	24.78
1	0.05	0.25	0 - 20	*	2	—	36.50+
3	0.05	0.25	0 - 40	0.2108	4	80.50	6.32
4	0.05	0.25	0 - 30	0.9595	3	12.55	28.79
1	0.05	0.125	0 - 20	*	2	—	25.0 +
5	0.10	4.00	0 - 40	3.6468	4	8.16	109.40
6	0.10	4.00	0 - 40	3.5982	4	7.78	107.95
5	0.10	2.00	0 - 30	2.2525	3	3.84	67.58
6	0.10	2.00	0 - 30	2.8870	3	14.44	86.61
5	0.10	1.00	0 - 20	*	2	—	37.00+
6	0.10	1.00	0 - 20	*	2	—	54.00+
5	0.10	0.50	0 - 20	*	2	—	27,50+
6	0.10	0.50	0 - 20	*	2	—	20.00+

.y - corrected calculated volumes CO_2 (μl).

\+ - b_{30} value derived by extrapolating line through two points.

* - line not calculated by regression analysis

Sb - standard error of regression coefficient (b) of line.

This tendency was more marked at the higher value of [E]. The mean b_{30} values, for a given value of [S], were also generally higher at the higher enzyme concentration. The coefficients of variation for the b_{30} values could be large and tended to increase as [S] decreased relative to [E].

b_{30} values calculated from a further five experiments in which [E] was reduced to 0.025 units/ml and the duration of the experiments was increased from forty to ninety minutes are given in Table 6. Here the production of CO_2 appeared, in most cases, to bear a rectilinear relationship to time over the ninety minutes of an experiment. This allowed regression analyses of CO_2 production on time to be performed for each combination of [E] and [S] used, in contrast to the previous experiments (Table 4). Additionally, in each experiment, the regression analyses were based on a larger number of values than previously. Despite these improvements it was seen that the errors of the slopes of the regression lines could be unacceptably large at the lower concentrations [S].

The means of the above b_{30} values are given in Table 7. The mean b_{30} values decreased and the coefficients of variation amongst the b_{30} values tended to rise as [S] was reduced for a given concentration [E]. At [S] equal to 0.5 m mol/l the coefficient of variation was very large. By comparison with the results given in Table 5, however, there was a tendency for the standard error of the mean b_{30} value to be lower at a given value [S] than previously.

From each of the five experiments the kinetic parameters K_M and V_{MAX} were calculated by the use of a double reciprocal plot (Lineweaver and Burk, 1934) of $\frac{1}{b_{30}}$ against $\frac{1}{[S]}$. These results are given in Table 8. The values for K_M were much higher than those expected for the reaction between AcCh and AcChE (0.1 - 1.0 m mol/l; Barlow, 1964) and they varied widely.

When the mean b_{30} values were plotted against [E] for each

Table 5

Analysis of b_{30} values from Table 4 at different concentrations AcChE [E] and AcCh [S] and at 37°C.

F.F.C [S] m mol/l	0.125	0.25	0.50	1.00	2.00	4.00
F.F.C[E] 0.05 units/ml						
Mean b_{30} value	25.00	23.87	27.46	32.13	52.36	52.51
Number of determinations.	1	3	3	4	4	2
Standard error of mean b_{30} value		9.05	13.18	8.09	2.95	6.90
Coefficient of variation for b_{30} values %	65.69	83.12	50.37	11.27		18.58
F.F.C [E] 0.10 units/ml						
Mean b_{30} value		23.75	45.50	77.10		108.68
Number of determinations		2	2	2		2
Standard error of mean b_{30} value		3.75	8.50	9.52		0.73
Coefficient of variation for b_{30} values %		22.33	26.42	17.45		0.94

F.F.C - final flask concentration.

Table 6

Results of manometric measurements of reaction velocity for the AcChE (E) catalysed hydrolysis of AcCh (S) at 37°C.

[E]= 0.025 units/ml

Expt.	[S] m mol/l	y. linearly related to time for period (mins)	Slope (b) of line y. on time	Number of points on line	Error of slope $\frac{(Sb)}{b}$ x 100%	b_{30} value $\mu l CO_2$/30 min.
5	8.00	0 - 90	1.0562	7	4.44	31.68
1	4.00	0 - 90	0.8849	6	9.11	26.55
2	4.00	0 - 90	0.9520	9	2.77	28.56
3	4.00	0 - 90	1.2141	9	4.80	36.43
5	4.00	0 - 90	0.9754	7	4.22	29.26
3	3.00	0 - 90	0.7440	8	5.54	22.32
4	3.00	0 - 90	0.8313	9	4.81	24.93
5	3.00	0 - 90	0.7258	7	9.53	21.78
1	2.00	0 - 90	0.7135	6	6.71	21.43
2	2.00	0 - 90	0.6834	8	6.54	20.49
3	2.00	0 - 90	0.9148	8	7.32	27.47
4	2.00	0 - 90	0.7077	9	9.58	21.24
5	2.00	0 - 90	0.8141	8	5.63	24.43
1	1.00	0 - 90	0.2558	6	7.82	7.67
2	1.00	0 - 60	0.4015	5	12.67	12.04
3	1.00	0 - 90	0.5629	9	7.94	16.89
4	1.00	0 - 90	0.4694	9	14.59	14.08
1	0.50	0 - 90	0.1672	6	45.10	5.01
2	0.50	0 - 90	0.0069	8	353.62	0.21
3	0.50	0 - 90	0.2790	9	13.41	8.37
4	0.50	0 - 50	0.3717	5	21.17	11.15
1	0.25	0 - 90	0.0552	6	112.01	1.66

.y - corrected calculated volumes CO_2 (μl)

Sb - standard error of regression coefficient (b) of line.

Table 7

Analysis of b_{30} values from Table 6 at a concentration AcChE [E] of 0.025 units/ml, various concentrations AcCh [S] and at 37°C.

F.F.C [S] m mol/l	0.25	0.50	1.00	2.00	3.00	4.00	8.00
Mean b_{30} value	1.66	6.19	12.67	23.01	23.01	30.20	31.68
Number of determinations	1	4	4	5	3	4	1
Standard error of mean b_{30} value		2.35	1.94	1.30	0.97	2.15	
Coefficient of variation amongst b_{30} values %		76.13	30.63	12.65	7.32	14.27	

F.F.C - final flask concentration.

Table 8

K_M and V_{MAX} values derived from plots of $\frac{1}{b_{30}}$ against $\frac{1}{[S]}$ for experiments 1 - 5 (Table 6)

Experiment	K_M (m mol/l)	V_{MAX} (μl CO_2/30 min.)
1	7.75	80.00
2	3.64	55.87
3	2.90	58.48
4	0.87	29.59
5	0.97	33.90
Mean Value	3.22	51.56
Standard error of mean	1.25	9.13
Coefficient of variation amongst results. %	87	40

concentration [S] , it was seen that a linear relationship over the three concentrations [E] obtained only when [S] was 4.0 m mol/l (Fig 5) It appeared that, with this exception, a prerequisite for a satisfactory enzyme assay - the rate of reaction being proportional to the amount of enzyme present (Witter,1963, Dixon and Webb, 1964) - had not been fulfilled.

It may have been that a concentration [E] equal to 0.10 unit/ml the enzyme was not saturated with substrate when the concentration of the latter was less than 4.0 m mol/l. Hence the apparent curvature of most of the graphs of b_{30} on [E] . Alternatively a rectilinear relationship between b_{30} and [E] might have been obscured at the lower concentrations [S] by the large standard errors of the mean b_{30} values when [E] was either 0.05 or 0.10 units/ml.

In order to attempt to reduce the errors of the regression lines from which the b_{30} values were derived and the errors amongst the b_{30} values at given concentrations [E] and [S] it was decided to repeat the experiments after reappraisal of the experimental technique. By this means it was also hoped that the relationships between b_{30} values and [E] would become less equivocal and that values of a more usual magnitude for K_M would be obtained.

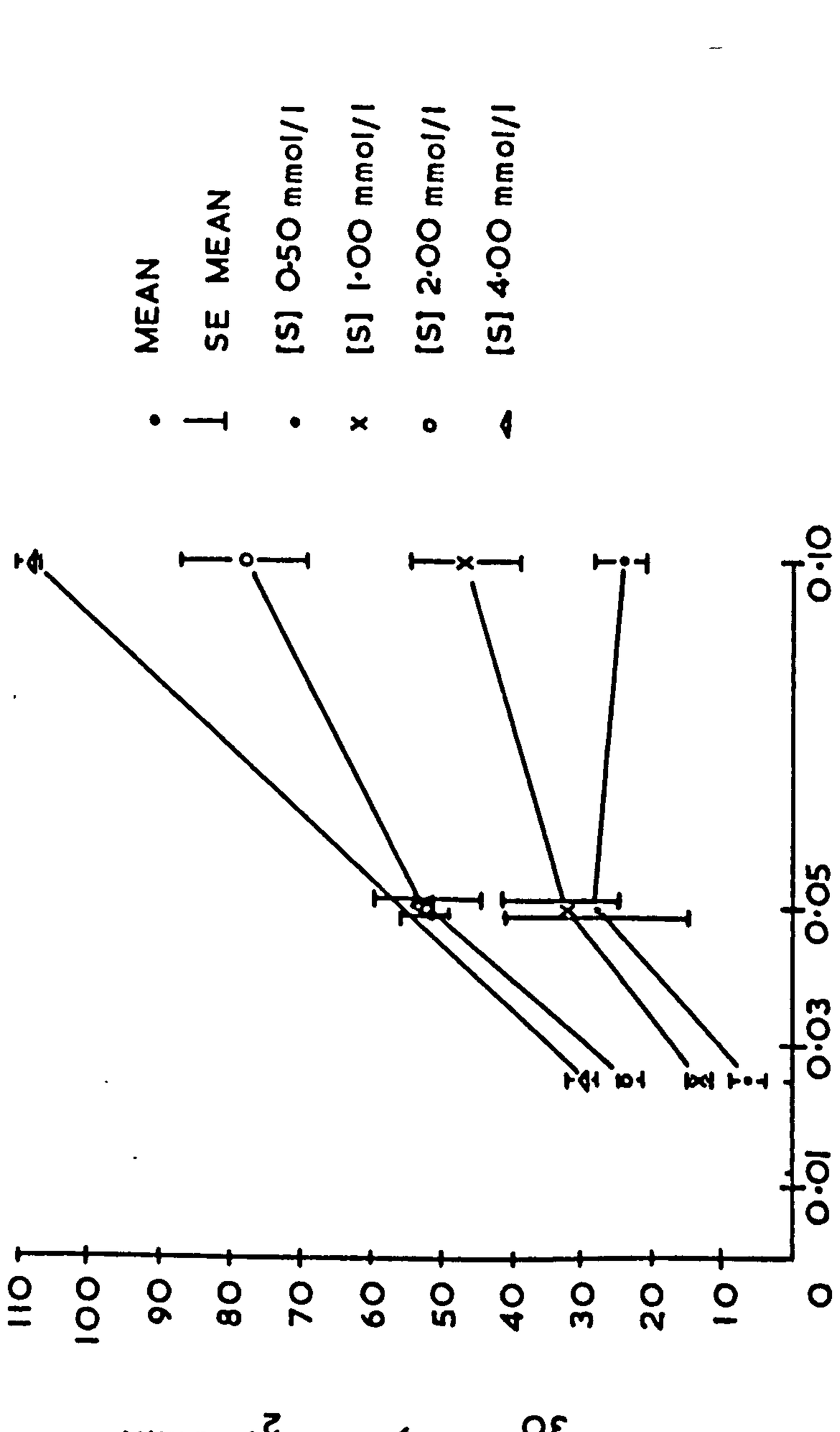


Fig 5.

Relationship between mean b_{30} values (from Tables 5 & 7) and enzyme concentrations [E] for the AcCh-AcChE reaction at 37°C.

various substrate concentrations [S]

Further investigation into the use of the Warburg manometric technique for the measurement of AcChE activity.

Modification of Experimental Technique.

The following modifications of technique were incorporated in the experiments which were performed in order to reinvestigate the measurement of AcChE catalysed hydrolysis of AcCh.

1. Preparation of reaction medium (B)

In the previous experiments, (B) was prepared in one litre quantities from stock solutions of salts and used over a period of time, being stored between experiments in the dark at $4^{o}C$. It was 'regassed' prior to each experiment.

It was decided that henceforth a fresh batch of (B) would be prepared prior to the start of each experiment using $NaHCO_3$ as solid salt and both NaCl and $MgCl_2 6H_2O$ from stock solutions. The pH of the resultant solution (B) was checked at the time of preparation prior to each experiment.

2. Use of reaction flask/manometer combinations.

An attempt was made to reduce any systematic error arising from the possible consistent assignation of any one flask/manometer combination to any given reaction system. This was achieved by assigning flask/manometer combinations to reaction systems (TB, EB, S_1B, ES_1B, S_2B, ES_2B etc.) by the use of random numbers.

It was also thought that the thermobarometer (TB) compensation would be more accurate if the flask (TB) contained 2.5ml (B) rather than the 2.5ml. deionised water as in the previous experiments.

3. Saturation of reagents with 5% CO_2/95%N_2

In order to more confidently achieve saturation of the reagents with 5% CO_2/95%N_2 it was decided that in 'loading' flasks with reagents, the solution (B) would be added last, just after it had been 'regassed', and just prior to the period of temperature and pH equilibration. Further, it was decided to extend the latter period from the fifteen

minutes used previously to twenty minutes.

4. Reading of manometers.

Results from the previous experiments performed with a F.F.C [E] of 0.25, 0.05 or 0.10 units/ml. (Tables 4 and 6) suggested that at the higher concentrations [E] and the lower concentrations [S], the initial linear reaction velocity (y) might only obtain over a period of 20-30 minutes. Here a ten minute interval between manometer readings was not thought to give enough results for adequate regression analysis. Hence the manometers were read over forty minutes and, to improve the accuracy of the subsequent regression analyses, the frequency of reading was every five minutes when F.F.C [S] was 4.0, 3.0 or 2.0 m mol/l or every two and a half minutes at the lower (1.0 and 0.5 m mol/l) concentrations [S].

Nine experiments were performed; two experiments at each pf the three concentrations [E] equal to 0.0125, 0.025 and 0.10 units/ml and three experiments with [E] equal to 0.05 units/ml. The experiments used a single batch of enzyme.

The experiments utilised five concentrations [S] namely 0.5, 1.0, 2.0, 3.0 and 4.0 m mol/l. This range of concentrations was chosen because previous experimehts (Tables 5 and 7) had suggested that reaction velocity might have been approaching a maximum when the F.F.C [E] was 0.025 or 0.05 units/ml and [S] was 4.0 m mol/l. Below a concentration [S] of 0.5 m mol/l it was thought that the reaction rate might have been too low for accurate determination when the lower concentrations [E] were used.

Results and Discussion.

Table 9 shows the slopes of the lines relating the production of CO_2 to time at all concentrations [E] and [S] in the nine experiments. These slopes were calculated by regression analysis in all cases and the errors of the slopes are also shown. The ability to perform such regression analyses reflected the adjustments made to thefrequency

Table 9

Results of manometric measurements of reaction velocity of the AcChE (E) catalysed hydrolysis of AcCh (S) at 37°C

Expt	[E] units/ml	[S] m mol/l	y' linearly related to time for period (mins).	Slope (b) of line y' on time	Number of points on line	Error of slope $\left(\frac{Sb}{b}\right)$ x 100%	b_{30} value μl CO_2/30 min.
1	0.0125	0.5	0 - 40	0.3093	16	13.71	9.28
2	0.0125	0.5	0 - 40	0.1231	16	42.16	3.70
1	0.0125	1.0	0 - 40	0.4018	13	8.61	12.06
2	0.0125	1.0	0 - 40	0.4894	16	9.58	14.68
1	0.0125	2.0	0 - 40	0.4484	4	11.57	13.46
2	0.0125	2.0	0 - 40	0.5713	8	13.78	17.14
1	0.0125	3.0	0 - 40	0.5209	7	10.16	15.62
2	0.0125	3.0	0 - 40	0.5215	8	14.07	15.64
1	0.0125	4.0	0 - 40	0.6592	5	4.00	19.77
4	0.025	0.5	0 - 40	0.5315	16	7.75	15.95
3	0.025	1.0	0 - 40	0.6517	8	9.45	19.55
4	0.025	1.0	0 - 40	0.6627	16	7.08	19.89
3	0.025	2.0	0 - 40	1.0097	8	5.94	30.28
4	0.025	2.0	0 - 40	0.8390	8	4.29	25.17
3	0.025	3.0	0 - 40	1.1092	8	4.59	33.28
4	0.025	3.0	0 - 40	1.0869	8	5.03	32,60
3	0.025	4.0	0 - 40	1.0919	8	3.17	32.75
4	0.025	4.0	0 - 40	1.0351	8	5.87	31.05
5	0.05	0.5	0 - 15	1.1901	6	9.13	35.70
6	0.05	0.5	0 - 15	1.9470	6	8.72	58.41
7	0.05	0.5	0 - 17.5	1.5375	7	5.78	46.13
5	0.05	1.0	0 - 15	1.9209	6	6.37	57.62
6	0.05	1.0	0 - 32.5	1.7999	13	5.58	54.00
7	0.05	1.0	0 - 20	1.9290	8	4.21	57.87

continued .....

Table 9 (continued)

Expt.	[E] units/ml	[S] m mol/l	y' linearly related to time for period (mins).	Slope (b) of line y' on time	Number of points on line	Error of slope $\frac{Sb}{b}$ x 100%	$\frac{b_{30}}{}$ value μl CO_2/30 min.
5	0.05	2.0	0 - 40	1.9117	8	3.18	57.35
6	0.05	2.0	0 - 40	2.0191	8	6.26	60.57
5	0.05	3.0	0 - 40	1.9627	7	2.33	58.88
6	0.05	3.0	0 - 40	2.1215	8	4.32	63.65
7	0.05	3.0	0 - 40	2.2226	8	3.46	66.68
5	0.05	4.0	0 - 40	1.9831	8	4.31	59.49
6	0.05	4.0	0 - 40	2.3518	8	4.25	70.56
7	0.05	4.0	0 - 40	2.3265	8	4.30	69.79
8	0.10	0.5	0 - 10	1.8288	4	13.21	54.87
9	0.10	0.5	0 - 10	2.6700	4	6.37	80.06
8	0.10	1.0	0 - 17.5	2.2044	7	7.63	66.13
9	0.10	1.0	0 - 15	2.6900	6	8.18	80.74
8	0.10	2.0	0 - 25	3.0248	5	3.77	90.74
9	0.10	2.0	0 - 20	4.1000	4	4.88	123.01
8	0.10	3.0	0 - 40	3.2024	8	3.75	96.08
9	0.10	3.0	0 - 40	3.3000	8	5.45	99.08
8	0.10	4.0	0 - 40	3.3185	8	2.35	99.56
9	0.10	4.0	0 - 40	3.6000	8	2.50	108.08

y' denotes corrected calculated volumes CO_2 (μl)

Sb - standard error of regression coefficient (b) of line.

of manometric readings at different concentrations [S] . This represented an improvement over some of the initial Warburg experiments (Table 4) where not enough manometric readings were obtained for regression analysis. The results of the initial Warburg experiments concerning the period of time over which CO_2 production bore a linear relationship to time for given concentrations [E] and [S] were, however, generally confirmed. It was concluded that when [E] was either 0.0125 or 0.025 units/ml and [S] was within the range 0.5 - 4.0 m mol/l then the linear relationship was maintained for at least forty minutes. When [E] was 0.05 units/ml this relationship may have ceased after fifteen minutes when [S] was 1.0 m mol/l or less. At [E] = 0.10 units/ml this relationship may have ceased after ten minutes when [S] was 0.5 m mol/l, after fifteen minutes when [S] was 1.0 m mol/l and after twenty minutes when [S] was 2.0 m mol/l. Table 9 also shows the b_{30} values calculated from the regression lines. The errors of the b_{30} values are given in Table 10 whilst the values K_M and V_{MAX} derived from six of the nine experiments are shown in Table 14.

Mean values for the errors of the regression line slopes at given concentrations [E] and [S] were derived from Table 9 . They were compared, where possible, with the corresponding mean values for error derived from Tables 4 and 6 by the application of 't' tests (Saunders and Fleming, 1966). The mean values for these errors, the standard errors of such means and the calculated values of 't' are shown in Table 11.

Table 11 showed that in all cases the mean values of the errors of the regression line slopes were numerically less in the nine later experiments than in the initial Warburg experiments. However only when [E] was 0.10 units/ml and [S] was 4.0 m mol/l was this reduction significant (P = 0.95). The general lack of significant reduction of mean error when [E] was 0.10 or 0.05 units/ml may well have reflected the large standard errors associated with some of the means in the

Table 10

Analysis of b_{30} values from Table 9 at different concentrations AcChE [E] and AcCh [S] and at 37°C

	[E]	[S] m mol/l				
	units/ml	0.5	1.0	2.0	3.0	4.0
Mean b_{30} value ($\bar{b}_{30}$)	0.0125	6.49	13.37	15.30	15.63	19.77
Number of determinations (N)		2	2	2	2	1
Standard error (S.E.) of $\bar{b}_{30}$		2.79	1.31	1.84	0.01	
Coefficient of variation (cv)% amongst b_{30} values.		60.80	13.86	17.01	0.09	
$\bar{b}_{30}$	0.025	15.95	19.72	27.73	32.94	31.90
N		1	2	2	2	2
S.E. $\bar{b}_{30}$			0.17	2.56	0.34	0.85
cv b_{30}%			1.22	13.03	1.46	3.77
$\bar{b}_{30}$	0.05	46.75	56.50	58.96	63.07	66.61
N		3	3	2	3	3
S.E. $\bar{b}_{30}$		6.56	1.25	1.61	2.27	3.57
cv b_{30}%		24.32	3.83	3.86	6.23	9.28
$\bar{b}_{30}$	0.10	67.47	73.44	106.88	97.58	103.82
N		2	2	2	2	2
S.E. $\bar{b}_{30}$		12.60	7.31	16.14	1.50	4.26
cv b_{30}%		26.40	14.07	21.35	2.17	5.80

Table 11

Comparison of mean values for the errors $\left(\frac{Sb}{b}\right)$ x 100% of slopes (b) of regression lines relating CO_2 produced by the AcChE (E) catalysed hydrolysis of AcCh (S) to time

mean values for errors derived from Tables 4, 6 and 9

[E] units/ml	[S] m mol/l	Mean value $\left(\frac{Sb}{b}\right)$ x 100% from Table 4 or 6. 1	Standard error of mean value	Mean value $\left(\frac{Sb}{b}\right)$ x 100% from Table 9. 2	Standard error of mean value	't' calculated between results 1 and 2	Degrees of freedom (df)
0.025	1.0	10.76	1.71	8.27	1.19	0.94	4
0.025	2.0	7.16	0.66	5.12	0.82	1.71	5
0.025	3.0	6.63	1.47	4.81	0.22	0.96	3
0.025	4.0	5.23	1.36	4.52	1.35	0.32	4
0.050	0.5	76.03	37.58	7.88	1.05	2.43	3
0.050	1.0	7.73	1.45	5.39	0.63	1.48	4
0.050	2.0	9.52	0.99	4.72	1.54	2.72	4
0.050	4.0	7.22	4.28	4.29	0.02	0.92	3
0.100	2.0	9.14	5.30	4.33	0.55	0.90	2
0.100	4.0	7.97	0.19	2.43	0.07	27.15	2

't' (theoretical) at P = 0.95; 4.30 (2df); 3.18 (3df); 2.78 (4df); 2.57 (5df).

initial experiments at these enzyme concentrations and the few degrees of freedom in the comparisons.

Examination of Table 10 showed that the b_{30} values were more precise (e.g. coefficient of variation <10%) over a greater range of the chosen substrate concentration when [E] was equal to 0.025 or 0.05 units/ml than when [E] was equal to 0.0125 or 0.10 units/ml. It was possible that when [E] was 0.0125 units/ml the reaction rates were too low to be determined with precision as evidenced by the relatively large errors associated with some of the regression lines from which these b_{30} values were derived. In contrast, when [E] was 0.10 units/ml, there may not have been adequate saturation of enzyme at lower substrate concentrations for precise determination of reaction rate as the release of CO_2 declined relatively quickly at these concentrations.

Comparison of Tables 5, 7 & 10 showed that at given concentrations [E] and [S], coefficients of variation amongst the b_{30} values were numerically less on most occasions (eight out of twelve) when the later results (Table 10) were compared with those of the initial Warburg experiments (Tables 5 and 7). Conversely the mean b_{30} values were numerically greater at all but one concentrations [E] and [S] in the later nine experiments although this difference was only significant (P = 0.95) in one comparison (Table 12).

The relationship between the mean b_{30} values and [E] was examined for each of the five chosen substrate concentrations (Fig 6). Particular attention was paid to the results obtained at the lower values of [S] when [E] was 0.10 units/ml. as the initial Warburg experiments had suggested that at such concentrations the relationship between mean b_{30} and [E] might diverge from linearity.

Two regression analyses of b_{30} on [E] were performed at each concentration [S]. One analysis included the mean b_{30} value when [E] was 0.10 units/ml. whilst the other analysis excluded this result. The errors of the regression lines calculated under these two

Table 12

Comparison of mean b_{30} values (Table 10) with the corresponding mean b_{30} values from the initial Warburg experiments (Table 5&7) by 't' test.

[E] units/ml	[S] m mol/l	Mean b_{30} value (Table 10)	Mean b_{30} value (Table 5 or 7)	Calculated value 't'	df
0.025	1.0	19.72	12.67	2.42	4
0.025	2.0	27.73	23.01	1.84	5
0.025	3.0	32.94	23.01	7.75	3
0.025	4.0	31.90	30.20	0.52	4
0.050	0.5	46.75	27.46	1.31	4
0.050	1.0	56.50	32.13	2.53	5
0.050	2.0	58.96	52.36	1.46	4
0.050	4.0	66.61	52.51	2.04	3
0.100	0.5	67.47	23.75	3.33	2
0.100	1.0	73.44	45.50	2.49	2
0.100	2.0	106.88	77.10	1.59	2
0.100	4.0	103.82	108.68	1.12	2

't' (theoretical) at P = 0.95 ; 4.30 (2df); 3.18 (3df); 2.78 (4df); 2.57 (5df).

circumstances may be compared in Table 13. This Table showed that in four of the five comparisons the error of the line was numerically less when the mean b_{30} value at $[E] = 0.10$ units/ml was excluded. Thus it appeared that for the range of AcCh concentrations 0.5 to 4.0 m mol/l assays with AcChE may be better performed within the range of enzyme concentrations 0.0125 to 0.05 units/ml than 0.0125 to 0.10 units/ml.

K_M and V_{MAX} values calculated from the results of six of the nine Warburg experiments are given in Table 14. They were obtained from the double reciprocal plot of $\frac{1}{b_{30}}$ on $\frac{1}{[S]}$ in those experiments where these parameters, by inspection, were rectilinearly related over the range of substrate concentrations employed. It will be seen that, as expected, the V_{MAX} values increased with increase in $[E]$. The K_M values obtained were, in contrast with those previously reported (Table 8), of a more usual order (0.1 - 1.0 m mol/l) for the interaction between AcCh and AcChE.

The results of the Warburg experiments reported above gave information concerning the interrelationships between the relative concentrations $[E]$ and $[S]$ and the frequency and duration of manometric readings required for the determination of AcChE activity. Numerically the errors of slopes of regression lines of CO_2 production on time and the errors of the b_{30} values have shown general improvement compared with the errors in the initial Warburg experiments. However the few degrees of freedom involved in these comparisons and the large errors of many of the results from the initial experiments meant that the improvements could generally not be claimed as statistically significant. This lack of statistically validated improvement in error and literature claims that the accuracy and precision of cholinesterase Warburg determinations could be within limits of 1-3% (Augustinsson, 1957;1963;1971; Witter 1962;1963) led to an examination of the influence of certain factors on the errors associated with the results from such determinations.

Table 13

AcChE (E) catalysed hydrolysis of AcCh (S)

Errors $\left(\frac{Sb}{b}\right)$ x 100% of regression lines relating mean b_{30} values to [E] at stated concentrations [S]

[E] = 0.0125; 0.025; 0.050 and 0.100 units/ml.

[S] m mol/l	Error $\left(\frac{Sb}{b}\right)$ x 100% when result at [E] = 0.10 units/ml included.	Error $\left(\frac{Sb}{b}\right)$ x 100% when result at [E] = 0.10 units/ml excluded.
0.5	18.04	10.73
1.0	23.39	19.93
2.0	4.03	5.36
3.0	11.57	3.54
4.0	10.17	8.15

Table 14

K_M and V_{MAX} values derived from plots of $\frac{1}{b_{30}}$ against $[\frac{1}{S}]$ for experiments shown in Table 9.

[E] units/ml	K_M (m mol/l)	V_{MAX} (μ l CO_2 produced/30mins).
0.0125	0.54	19.01
0.0250	0.63	35.21
0.0500	0.38	73.53
0.0500	0.31	74.63
0.1000	0.53	111.11
0.1000	0.24	114.94

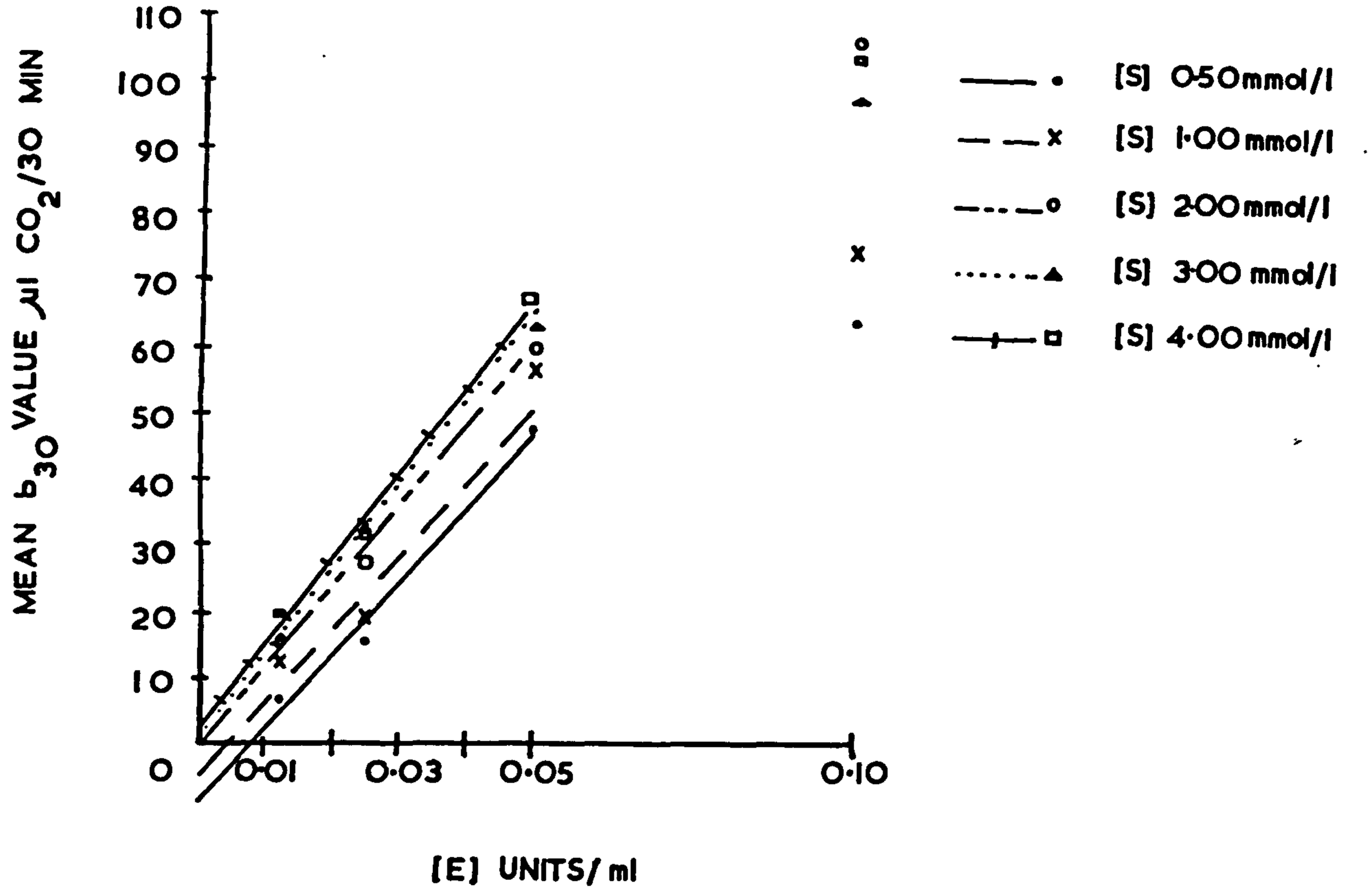


Fig 6.

Relationship between mean b_{30} values (from Table 10) and enzyme concentrations [E] at various substrate concentrations [S] for the AcCh-AcChE reaction at 37°C.

<u>An examination of the influence of some factors on the results and associated errors from the determinations of AcChE activity by Warburg manometry.</u>

An initial assessment of the accuracy and precision with which determinations of AcChE activity may be achieved by the use of Warburg manometry was made in the experiments previously reported. This assessment involved calculations of the errors both of b_{30} values and of the slopes of regression lines from which the b_{30} values were derived. It was shown that such errors could be high but that results with lower errors were to be expected with the use of certain concentrations $[E]$ and $[S]$. Therefore it was decided that an assessment of the influence of certain factors on the results and associated errors from the manometric determination of AcChE activity using AcCh as substrate might be best made by a study of results generated with selected single, fixed, concentrations $[E]$ and $[S]$. A fresh batch of enzyme was used for this purpose.

The chosen concentrations $[E]$ and $[S]$ were 0.05 units/ml and 2.0 m mol/l F.F.C respectively. Past results had shown that both the magnitude of the b_{30} value and the errors associated with the results at these concentrations were of a reasonable order. (Tables 5 and 10). Too high a concentration $[S]$, for the chosen concentration $[E]$, was avoided to prevent the results being complicated by enzyme inhibition effected by excess substrate.

To make doubly sure that the reagent solutions were fully saturated with 95% N_2/5% CO_2 the following precautions were adopted prior to the period of temperature and pH equilibration. The reaction medium (B) was 'gassed' with 5% CO_2/95% N_2 for a fixed period of five minutes prior to being used to prepare the required dilutions $[E]$ and $[S]$. Further, each of the solutions (B),(E), and (S) were also gassed for a fixed period of two minutes before being loaded into reaction flasks. The remainder of the experimental

protocol was that of the preceding nine experiments (pages 68 to 79).

On each of eight (not consecutive) days three experiments were performed each utilising a set of reaction systems comprising TB, EB, SB and ESB components. Three b_{30} values, one from each set, were therefore generated on each day. Thus the b_{30} values and the slopes of the regression lines from which they were derived on a single day could be analysed and compared with the results derived on different days. The study was also designed to test whether or not the order of reading the manometers of the TB, EB, SB and ESB components within each set of reaction systems affected the results.

The contribution that the results from the 'blank's (EB) and (SB) made to the calculated reaction velocity and its error was examined. Additionally information was sought concerning the relative merits of using the b_{30} value as the expression for the reaction velocity, as in the previous experiments, or the slope of the corresponding regression line from which it had been derived.

The experimental design is given in Table 15. In the design manometer/flask systems were assigned both to functions (TB,EB, etc) as before, and to sets of reaction systems by random numbers. In addition the order of reading manometers within a given set was altered over succeeding experiments. By this means in each of sets 2 and 3 the alteration in the reading order involved a repetition of a Latin Square design over the period of the experiments. In set 1, the reading order was the same in all experiments.

Table 15

Design of experiments to investigate the results from the AcChE catalysed hydrolysis of AcCh at a fixed concentration of both enzyme [E] and substrate [S] .

Thermobarometer (TB) = A

Enzyme/buffer blank (EB) = B

Substrate/buffer blank (SB) = C

Enzyme catalysed reaction (ESB) = D

[E] = 0.05 units/ml F.F.C

[S] = 2.0 m mol/l

DAY	Sets of Reaction systems. 1 | 2 | 3 Reading order for manometers		
1	A B C D	C A D B*	D C B A*
2	A B C D	D C B A	B D A C
3	A B C D	B D A C	A B C D
4	A B C D	A B C D	C A D B
5	A B C D	C A D B*	D C B A*
6	A B C D	D C B A	B D A C
7	A B C D	B D A C	A B C D
8	A B C D	A B C D	C A D B

* Latin Square design.

Results.

In all twenty-four experiments carried out on the eight different days, the production of CO_2 was, by inspection, rectilinear with respect to time for the whole of the forty minutes during which the reaction was measured. This was in agreement with the earlier work at the same concentrations [E] &[S] (Tables 4 and 9).

Table 16 shows the values for the errors of the individual calculated regression lines relating CO_2 production to time. These values are shown in relation to day of experiment, sets of reaction systems, and the order of reading manometers within sets of reaction systems. The mean value for all the errors was 4.29% (range 1.82% - 8.45%).

The b_{30} values derived from these regression lincs are given in Table 17 again in relation to day of experiment and sets of reaction systems. Results of 't' tests performed between the mean b_{30} values obtained on different days are shown in Table 18and between the mean b_{30} values obtained in different sets of reaction systems in Table 19 The mean b_{30} values were significantly different in ten of the twenty-eight 'between-day' comparisons. There were no significant differences in the mean b_{30} values obtained in the different sets of reaction systems. It will also be seen(Table 20) that there were no significant differences between the mean b_{30} values obtained when the order of reading manometers within sets of reaction systems was taken into account.

An analysis of variance was carried out to compare the variance in b_{30} values obtained 'within experimental days' with the variance in b_{30} values 'between days'. The method of analysis given by Saunders and Fleming (1966) was used. The 'between days' variance was 120.85 and 'within days' variance 21.67, the ratio 'F' being 5.58 for 7 and 16 degrees of freedom (df) respectively. The theoretical value of 'F' for 7 and 16 df at P=0.95 was 2.66. Thus the 'between days'

Table 16

Reaction between AcCh (2.0 m mol/l) and AcChE 0.05 units/ml at 37°C

Errors of individual regression lines used to derive b_{30} values shown in Table 17

$$\text{Error} = \left(\frac{Sb}{b}\right) \times 100\%$$ where b = slope of regression line
Sb = standard error of regression coefficient.

DAY	Sets of reaction systems			Mean error %
	1	2	3	
1	3.09	3.79	4.38	3.75
2	2.99	3.10	5.31	3.80
3	4.79	4.16	8.45	5.80
4	4.91	2.18	3.09	3.39
5	3.80	4.01	4.38	4.06
6	6.29	3.48	8.41	6.06
7	3.44	2.52	6.39	4.12
8	1.82	3.56	4.63	3.34
Mean error	3.89	3.35	5.63	

Reading order of manometers within sets of reaction systems.

ABCD	CADB	DCBA	BDAC
3.09	3.79	4.38	5.31
2.99	3.09	3.10	4.16
4.79	4.01	4.38	8.41
8.45	4.63	3.48	2.52
4.91			
2.18			
3.80			
6.29			
3.44			
6.39			
1.82			
3.56			
Mean error			
4.31	3.88	3.84	5.10

Mean error for all regression lines = 4.29%

Coefficient of variation amongst all errors = 39.68%

A = Thermobarometer (TB) B = Enzyme/buffer blank (EB)

C = Substrate/buffer blank (SB) D = Enzyme catalysed reaction (ESB)

Table 17

b_{30} values (μ l CO_2/30 min.) obtained from interaction between AcCh (2.0 m mol/l) and AcCh E (0.05 units/ml) at 37°C

DAY	Set of reaction systems			Mean b_{30} value	Coefficient of variation %	Column
	1	2	3			
1	52.87	60.49	64.74	59.37	10.13	1
2	53.12	57.03	52.09	54.08	4.82	2
3	61.54	55.01	59.85	58.80	5.76	3
4	47.79	47.92	54.13	49.95	7.25	4
5	59.75	55.41	61.61	58.92	5.40	5
6	50.03	43.49	46.81	46.78	6.99	6
7	35.20	40.53	51.19	42.31	19.24	7
8	55.44	60.72	51.94	56.03	7.89	8
Mean b_{30}	51.97	52.58	55.30			
Coefficient of Variation %	15.75	14.59	11.05			
Column	9	10	11			

Mean b_{30} value from all results shown in Table = 53.28

Coefficient of variation amongst all results in Table = 13.52%

Table 18

Reaction between AcCh (2.0 m mol/l) and AcChE (0.05 units/ml) at 37°C

Results of 't' tests performed on b_{30} values obtained on different days and listed in Table 17 (Columns 1-8)

P = 0.95 4 degrees of freedom (df) t (theoretical) = 2.78

t test between columns	t(calc).	Difference significant	t test between columns	t(calc).	Difference significant
1 and 2	1.40	No	3 and 4	3.09	Yes
1 and 3	0.14	No	3 and 5	0:05	No
1 and 4	2.32	No	3 and 6	4.42	Yes
1 and 5	0.11	No	3 and 7	3.24	Yes
1 and 6	3.19	Yes	3 and 8	0.86	No
1 and 7	2.92	Yes	4 and 5	3.22	Yes
1 and 8	0.77	No	4 and 6	1.22	No
2 and 3	1.91	No	4 and 7	1.48	No
2 and 4	1.60	No	4 and 8	1.84	No
2 and 5	2.04	No	5 and 6	4.61	Yes
2 and 6	3.03	Yes	5 and 7	3.29	Yes
2 and 7	2.39	No	5 and 8	0.92	No
2 and 8	0.66	No	6 and 7	0.88	No
			6 and 8	2.92	Yes
			7 and 8	2.57	No

Table 19

Reaction between AcCh (2.0 m mol/l) and AcChE (0.05 units/ml)

Results of 't' tests performed on b_{30} values obtained in different sets of reaction systems and listed in Table 17 (columns 9-11)

P = 0.95 14 df t (theoretical) = 2.14

t test between columns	t(calc)	Difference significant
9 and 10	0.15	No
9 and 11	0.92	No
10 and 11	0.78	No

Table 20

b_{30} values (µl CO_2/30 mins) obtained from AcCh/AcChE interaction and related to the order of reading manometers within sets of reaction systems. b_{30} values previously given in Table 17

A = Thermobarometer (TB) B = Enzyme/buffer blank (EB)
C = Substrate/buffer blank(SB) D = Enzyme catalysed reaction (ESB)

Reading orders of manometers within sets of reaction systems.

ABCD	CADB	DCBA	BDAC
52.87	60.49	64.74	52.09
53.12	54.13	57.03	55.01
61.54	55.41	61.11	46.81
59.85	51.94	43.49	40.53
47.79			
47.92			
59.75			
50.03			
35.20			
51.19			
55.44			
60.72			
Mean b_{30} 52.95	55.49	56.72	48.61
Coefficient of variation % 14.10	6.54	16.52	13.10
Column 1	2	3	4

t tests between columns	t(calc)	t(theo)	df	Difference Significant P=0.95
1 and 2	0.64	2.14	14	No
1 and 3	0.82	2.14	14	No
1 and 4	1.94	2.14	14	No
2 and 3	0.24	2.45	6	No
2 and 4	1.88	2.45	6	No
3 and 4	1.43	2.45	6	No

variance was significantly greater than the 'within days' variance.

By contrast there was no significant difference in the variances of the b_{30} results obtained within and between different sets of reaction vessels. (F(calculated) 1.77; F(theoretical), P =0.95, 2 and 21 df = 3.47).

't' tests showed that neither the mean of all the b_{30} values from the twenty-four experiments nor the means of the b_{30} values calculated in previous experiments (Table5 and 10) using the same concentrations [S] and [E] but modifications of the same experimental technique were significantly different (P = 0.95). The results of this comparison are given in Table 21.

Influence of the use of compensatory 'blanks' on the results.

It has been seen that each calculation of reaction velocity has involved the use of results from three 'blanks' (TB,SB,EB) in equations:-

$$y\ (\mu l\ CO_2) = \text{Vol ESB} - \text{Vol EB} - \text{Vol SB} + \text{Vol TB}$$

It was decided to ascertain whether the use of such 'blanks' contributed significantly to the calculated reaction velocity or to the errors associated with the result.

Accordingly the production of CO_2 at given times, in the twenty-four experiments, was recalculated using the following simpler equation in which the use of two of the 'blanks' was eliminated.

$$y\ (\mu l\ CO_2) = \text{Vol ESB} - \text{Vol TB}$$

Regression analyses of the resulting values, y, on time were again performed and b_{30} values calculated from the regression lines

Values for the errors of these regression lines and for the resultant b_{30} values are tabulated according to the day of experiment and the sets of reaction systems (Tables 22and 23).

The mean value for all the regression line errors shown in Table 22 was 3.83% (range 1.87% - 5.70%). A 't' test carried out between this mean and the mean of all the errors shown in Table 16gave a value of t of 1.11 (46df) and showed no significant difference between the means

Table 21

Comparison of b_{30} values (μl CO_2/30 mins) obtained from the interaction of AcCh (2.0 m mol/l)with AcChE (0.05 units/ml) using different modifications of the same experimental technique.

For details of the different techniques see the appropriate sections of the text.

Result previously given in	Table 5	Table 10	Table 17
Mean b_{30}	52.36	58.96	53.28
No. of experiments.	4	2	24
Coefficient of variation amongst b_{30} values %	11.27	3.86	13.52
Columns	1	2	3

Calculated 't' value between columns	t(calc)	df	t(theo) P =0.95	Difference Significant
1 and 2	1.46	4	2.78	No
1 and 3	0.24	26	2.06	No
2 and 3	1.09	24	2.06	No

Table 22

Reaction between AcCh (2.0 m mol/l) and AcChE (0.05 units/ml) at 37°C

Errors of individual regression lines used to derive b_{30} values shown in Table 23

Error = $\left(\frac{Sb}{b}\right)$ x 100% where b = Slope of regression line

Sb = Standard error of regression coefficient.

DAY	Sets of reaction systems			Mean error %
	1	2	3	
1	4.11	2.92	3.07	3.37
2	3.57	2.87	5.04	3.83
3	3.36	4.21	3.29	3.62
4	1.87	1.89	2.39	2.05
5	3.71	3.89	4.19	3.93
6	4.99	2.70	5.57	4.42
7	5.28	3.65	4.25	4.39
8	5.70	4.63	4.86	5.06
Mean error	4.07	3.35	4.08	

Mean error for all regression lines = 3.83%

Coefficient of variation amongst all errors 28.56%

Table 23

$\underline{b_{30}}$ values (μl CO_2/30 mins) obtained from interaction between AcCh (2.0 m mol/l) and AcChE (0.05 units/ml) and from use of the relationship y = Vol ESB - Vol TB at 37°C.

y is volume CO_2 (μl) measured at given times during each experiment.

DAY	Set of Reaction Systems 1	2	3	Mean b_{30} value	Coefficient of variation %	Column
1	49.84	55.74	54.24	53.27	5.76	1
2	52.65	54.99	55.32	54.32	2.68	2
3	47.81	53.16	52.36	51.11	5.65	3
4	46.76	44.40	54.87	48.68	11.28	4
5	49.86	58.22	58.02	55.37	8.62	5
6	43.20	39.39	50.93	44.51	13.21	6
7	29.31	33.32	44.24	35.62	21.69	7
8	44.72	53.89	44.17	47.59	11.47	8
Mean b_{30}	45.52	49.14	51.77			
Coefficient of variation%	15.84	18.31	9.87			
Column	9	10	11			

Mean b_{30} from all results shown in Table = 48.81

Coefficient of variation amongst all results in Table = 15.23%

(P = 0.95, t (theo.) 2.01). It was noted, however, that when the twenty-four pairs of corresponding errors from Tables 16 and 22 were examined the regression line errors were numerically less when the simpler equation was used for CO_2 production in sixteen of the twenty-four pairs. Thus it was possible that the lack of significant differences shown by the 't' test between the mean errors may have reflected the masking of differences by the variation in errors both 'within' and 'between' days. To check this possibility the null hypothesis was proposed that the differences between the corresponding regression line errors could be attributed to chance and that they were drawn from a population of differences with a universal mean difference (μ) of zero. The hypothesis was tested by calculating a value of 't' using the expression

$$t = \frac{\bar{x} - \mu}{S\bar{x}}$$

where $\bar{x}$ was the mean difference in the pairs of regression line errors

and $S\bar{x}$ was the standard error of this mean difference. By comparison of the calculated value 't' with values given in Tables (Geigy, 1970) the probability of the null hypothesis being correct was found. The calculated value of 't' was 1.25 and the probability that the differences between the corresponding regression line errors were due to chance was $>$ 5% (23df). It was therefore confirmed that the errors in the regression lines derived from calculations of CO_2 production by the two formulae were not significantly different.

't' tests were performed between the mean b_{30} values calculated for different days and different sets of reaction systems and shown in Table 23 . The results of the 't' tests are in turn shown in Tables 24 and 25 . It was seen that in only five of the twenty-eight 'between-day' comparisons were the mean b_{30} values significantly different. None of the mean b_{30} values from the different sets of reaction systems were significantly different.

Table 24

Reaction between AcCh(2.0m mol/l) and AcChE (0.05 units/ml) at 37°C

Results of 't' tests performed on b_{30} values obtained on different days and from use of the relationship y = Vol ESB - Vol TB and listed in Table 23 (columns 1-8)

P = 0.95, 4 df . t (theoretical) = 2.78

t test between columns	t(calc).	Difference Significant	t test between columns	t(calc).	Difference Significant
1 and 2	0.53	No	3 and 4	0.68	No
1 and 3	0.89	No	3 and 5	1.32	No
1 and 4	1.27	No	3 and 6	1.75	No
1 and 5	0.64	No	3 and 7	3.25	Yes
1 and 6	2.29	No	3 and 8	0.99	No
1 and 7	3.68	Yes	4 and 5	1.59	No
1 and 8	1.57	No	4 and 6	0.90	No
2 and 3	1.72	No	4 and 7	2.38	No
2 and 4	1.72	No	4 and 8	0.24	No
2 and 5	0.36	No	5 and 6	2.48	No
2 and 6	2.81	Yes	5 and 7	3.77	Yes
2 and 7	4.19	Yes	5 and 8	1.86	No
2 and 8	2.06	No	6 and 7	1.58	No
			6 and 8	0.67	No
			7 amd 8	2.19	No

Table 25

Reaction between AcCh (2.0 m mol/l) and AcChE (0.05 units/ml) at 37°C

Results of 't' tests performed on b_{30} values obtained in different sets of reaction systems from use of the relationship

y = Vol ESB - Vol TB and listed in Table 23 (Columns 9-11)

P = 0.95 14 degrees of freedom t(theoretical) = 2.14

t test between columns	t (calc).	Difference significant
9 and 10	0.89	No
9 and 11	2.00	No
10 and 11	0.72	No

An analysis of variance amongst the b_{30} values in Table 23 showed that the variance of results 'between days' was significantly greater (P=0.95) than the variance of results 'within days' ('F' (calculated) = 5.09,'F' (theoretical) 7, 16 df = 2.66). The variances of the results 'between sets' of reaction systems were not significantly different (P=0.95) from the variances of results 'within sets' (F(calculated) 1.52; F(theoretical) 3.47; 2, 21 df).

A 't' test between the means of all the b_{30} values derived by use of the full and simpler formulae for calculating the volume CO_2 produced at given times gave a value of 't' of 2.12 and showed a significant difference (P = 0.95; 46 df, t(theo.) = 2.01). The mean b_{30} value derived from experiments where the simpler formula was used was significantly less than when the full formula was employed. This result was confirmed by again proposing the null hypothesis that the differences between corresponding b_{30} values were due to chance and were drawn from a population of differences with a universal mean of zero. Calculation of a value of 't' (4.72, 23 df) indicated that the probability that the differences were due to chance was less than 0.1% and thus the differences were highly significant.

When similar null hypotheses were proposed in respect of differences of b_{30} values calculated on the same day it was found that only on two of the eight days (days 7 and 8) was the difference significant. On day 7 the probability that the difference was due to chance lay between 0.1% and 0.5% (t,16.55; 2 df). On day 8 the same probability lay between 1 and 2% (t=7.20; 2 df).

An 'F' test between the variances of all the b_{30} values derived both by the full and simpler formulae for reaction velocity gave a value of 1.07. When compared with a tabulated value of 2.00 for 23 and 23 df and at P= 0.95, it was seen that the variances of the b_{30} values derived by the two calculations were not significantly different.

A further assessment of the influence of the 'blanks' SB and EB on the calculated b_{30} values was made. Calculations of CO_2 volume changes attributable both to the enzyme preparation (Vol EB - Vol TB) and due to the spontaneous hydrolysis of the substrate (Vol SB - Vol TB) were made for each of the twenty-four experiments. Regression analyses of the respective volume changes on time were performed and the errors of the resultant regression lines tabulated (Table 26). Additionally the corresponding calculated volume changes over thirty minutes were tabulated (Table 27).

The mean value for the errors of all the regression lines relating volume changes due to the enzyme preparation to time was 43.57% (range 8.17% - 145.47%).

The mean value for the errors of all the regression lines relating volume changes due to spontaneous hydrolysis to time was 152.91% (range 10.90% - 749.59%).

The mean volume change due to the enzyme preparation calculated from all the regression lines was -3.80 µl, the overall coefficient of variation amongst the results being 64.74%.

The corresponding mean volume change due to spontaneous hydrolysis was -0.62 µ l, the overall coefficient of variation amongst the results being 530.97%.

<u>The use of the b_{30} value or the slope of the regression line relating CO_2 production to time as alternative expressions of reaction velocity.</u>

The results from the Warburg experiments presently under consideration were examined to ascertain whether or not the b_{30} value was a better expression for reaction velocity than the slope of the corresponding regression line relating CO_2 production to time. An evaluation of the relative merits of these two expressions for reaction velocity was made by a comparison of their errors. The slopes of the regression lines (b) and the standard errors of their regression

Table 26

Errors in individual regression lines relating compensated volume changes Vol (EB÷TB) due to enzyme preparation to time.

Vol. EB = Volume change in Enzyme/buffer blank (μl)

Vol. TB = Volume change in thermobarometer (μl)

Error = $\left(\frac{Sb}{b}\right)$ x 100% where b = slope of regression line

Sb = standard error of regression coefficient

DAY	Sets of Reaction Systems			Mean error %
	1	2	3	
1	59.55	25.69	17.84	34.36
2	23.20	50.28	143.73	72.40
3	19.14	12.13	52.24	27.84
4	75.97	29.13	38.85	47.98
5	8.17	145.47	41.35	65.00
6	33.46	40.28	72.53	48.76
7	36.85	28.33	34.26	33.15
8	19.60	25.27	12.47	19.11
Mean error %	34.49	44.57	51.66	

Errors in individual regression lines relating compensated volume changes (Vol SB - Vol TB) due to spontaneous hydrolysis of substrate to time.

Vol. SB = Volume change in Substrate/buffer blank (μl)

DAY	Sets of Reaction Systems			Mean error %
	1	2	3	
1	66.75	59.13	58.90	61.59
2	46.75	566.87	59.50	224.37
3	25.03	36.47	54.77	38.76
4	749.59	115.42	50.66	305.22
5	45.69	18.81	103.76	56.09
6	95.26	255.50	10.90	120.55
7	27.52	44.22	36.59	36.11
8	42.20	399.85	699.76	380.60
Mean error %	137.35	187.03	134.36	

Table 27

Volume changes due to enzyme preparation (Vol EB - Vol TB, µl) over thirty minutes.

Vol EB = Volume change in enzyme/buffer blank (µl)

Vol TB = Volume change in thermobarometer (µl)

DAY	Sets of reaction systems			Mean change (µl)	Coefficient of variation %
	1	2	3		
1	-2.09	-3.89	-7.79	-4.59	63.48
2	-2.55	-2.36	+1.36	-1.11	186.31
3	-5.47	-5.02	-2.01	-4.17	45.15
4	-0.96	-2.85	-2.82	-2.21	48.99
5	-7.95	-1.11	-2.14	-3.73	98.78
6	-4.96	-3.74	-3.09	-3.93	24.16
7	-1.45	-5.52	-3.62	-3.53	57.69
8	-7.57	-5.58	-8.00	-7.05	18.31
Mean change (µl)	-4.13	-3.76	-3.51		
Coefficient of variation %	66.58	42.45	88.04		

Volume changes due to spontaneous hydrolysis (Vol SB - Vol TB, µl) over thirty minutes.

Vol SB = Volume change in substrate/buffer blank(µl)

DAT	Sets of reaction systems			Mean change (µl)	Coefficient of variation %
	1	2	3		
1	-1.27	-0.87	-2.70	-1.61	59.63
2	+2.10	+0.31	+1.87	+1.43	68.26
3	-8.27	+3.18	-5.49	-3.53	169.34
4	-0.09	-0.69	+3.55	+0.92	248.50
5	-1.94	+3.92	-1.45	+0.18	1840.22
6	-1.87	-0.36	+7.21	+1.66	293.10
7	-4.43	-1.69	-3.31	-3.14	43.83
8	-3.17	+0.31	+0.23	-0.88	226.60
Mean change (µl)	-2.37	+0.51	-0.01		
Coefficient of variation %	130.21	387.75	36591.89		

coefficients (Sb) were already known. So, too, were the corresponding b_{30} values. Accordingly the errors of the b_{30} values were calculated.

The b_{30} value was the calculated CO_2 volume produced at thirty minutes corrected for the calculated volume of CO_2 at zero time.

Thus b_{30} = Vol. $CO_{2(30\ min.)}$ - Vol $CO_{2(0\ min.)}$

= $y_{(30\ min.)}$ - c where c was the y intercept of the regression line at zero time.

The chosen expression for the error of a give b_{30} value (Sb_{30}) was:-

$$Sb_{30} = \sqrt{S^2c + S^2y_{(30\ min.)}}$$ where S^2c was the variance of the intercept of the regression line on the y axis and $S^2y_{(30\ min.)}$ was the variance of the value y at thirty minutes.

In turn S^2c and $S^2y_{(30\ min.)}$ were calculated by use of the following equations (Geigy,1970):-

$$S^2c = S^2b \quad \left(\frac{\sum x}{N}\right)$$

$$S^2y_{(30\ min.)} = S^2b\left[\sum x^2 - \left(\frac{\sum x}{N}\right)^2 + (30 - \bar{x})^2\right]$$ where the values x were the times at which CO_2 production was calculated and N was the number of points on which a regression analysis was based.

The corresponding ratios $\frac{Sb}{b}$ and $\frac{Sb_{30}}{b_{30}}$ were then compared (Table 28). In all cases the ratio $\frac{Sb_{30}}{b_{30}}$ was numerically less than the corresponding ratio $\frac{Sb}{b}$. The null hypothesis that the differences in these corresponding ratios were due to chance was proposed, the differences being drawn from a population of differences with a universal mean of zero. The null hypothesis was tested by the calculation, from the differences, of a value for 't'. From the twenty-four sets of differences the calculated value of 't' was 10.93 showing that the probability of the differences being due to chance was $< 0.1\%$ and that the differences were highly significant.

When the differences were tested in respect of results obtained

Table 28

Corresponding ratios $\frac{Sb}{b}$ and $\frac{Sb_{30}}{b_{30}}$ for each of twenty-four Warburg experiments measuring the hydrolysis of AcCh (2.0 m mol/l) catalysed by AcChE (0.05 units/ml) at 37°C.

b = slope of regression line relating CO_2 production to time.
Sb = standard error of regression coefficient.
b_{30} = b_{30} value (μl CO_2 produced in thirty minutes).
Sb_{30} = error of b_{30} value (see text).

DAY	Expt.	b	Sb	$\frac{Sb}{b}$	b_{30}	Sb_{30}	$\frac{Sb_{30}}{b_{30}}$
1	1	1.7626	0.0544	0.030863	52.87	1.5558	0.029427
	2	2.0167	0.0764	0.037883	60.49	2.1850	0.036120
	3	2.1580	0.0946	0.043836	64.74	2.7057	0.041790
2	1	1.7703	0.0530	0.029938	53.12	1.5223	0.028660
	2	1.9011	0.0590	0.031034	57.03	1.6946	0.029710
	3	1.7365	0.0922	0.053095	52.09	2.6481	0.050837
3	1	2.0513	0.0938	0.047920	61.54	2.8233	0.045877
	2	1.8337	0.0763	0.041610	55.01	2.1914	0,039836
	3	1.9950	0.1685	0.084460	59.85	4.8398	0.080865
4	1	1.5929	0.0623	0.039111	47.79	1.7894	0.037442
	2	1.5975	0.0348	0.021784	47.92	0.9995	0.020858
	3	1.8045	0.0557	0.030867	54.13	1.5997	0.029553
5	1	1.9914	0.0756	0.037963	59.75	2.1714	0.036340
	2	1.8470	0.0740	0.040064	55.41	2.1255	0.038359
	3	2.0536	0.0899	0.043776	61.61	2.5822	0.041911
6	1	1.6675	0.1049	0.062908	50.03	3.0130	0.060224
	2	1.4495	0.0504	0.034770	43.49	1.4476	0.033285
	3	1.5605	0.1313	0.084140	46.81	3.7712	0.080564
7	1	1.1731	0.0403	0.034353	35,20	1.1575	0.032883
	2	1.3510	0.0341	0.025240	40.53	0.9791	0.024157
	3	1.7061	0.1090	0.063888	51.19	3.1308	0.061160
8	1	1.8483	0.0336	0.018178	55.44	0.9647	0.017400
	2	1.9720	0.0702	0.035598	60.72	2.0163	0.033207
	3	1.7311	0.0801	0.046271	51.94	2.3007	0.044295

on different days the calculated values of 't' and the corresponding probabilities (P) that the results were due to chance were as follows:-

DAY	't'	P(2df)
1	8.10	$>1\%$ $<2\%$
2	0.41	$>5\%$
3	3.55	$>5\%$
4	4.96	$>2.5\%$ $<5\%$
5	19.89	$>0.1\%$ $<0.5\%$
6	3.48	$>5\%$
7	2.89	$>5\%$
8	2.90	$>5\%$

Thus the differences in the ratios from results on three of the eight days were significant, the ratio $\frac{Sb_{30}}{b_{30}}$ being less than $\frac{Sb}{b}$ on these occasions.

Experiments with inhibitors.

The Warburg manometric technique, as evolved in the present research, was applied to a limited investigation of cholinesterase inhibitors (I). The experiments involved the compound physostigmine sulphate as a known inhibitor and the compounds 2-methyl-4-phenethylamino -1,2,3 - benzotriazinium iodide (PMBI) and 4-benzylamino-2-n-propyl-1,2,3-benzotriazinium iodide (BnPBI) as potential inhibitors of the reaction between AcChE and AcCh.

Before the inhibitors were investigated the enzyme -substrate reaction was characterised by the determination of the kinetic parameters K_M and V_{MAX}. The mean values from four determinations of each of these parameters, with their standard errors were 0.73 ± 0.057 m mol/l and 69.15 ± 7.83 µl CO_2 per 30 min. respectively. The mean K_M value was of the appropriate magnitude for the reaction (Barlow,1964).

It was decided to investigate the effect of these compounds on the reaction between AcCh (2.0 m mol/l) and AcChE (0.05 units/ml). These concentrations were selected because past results had shown that they gave a reaction velocity which could be measured over forty minutes with acceptable precision and which was of sufficient magnitude to demonstrate the effect of an inhibitor.

Each of the compounds under investigation was tested at various concentrations [I] . For an experiment with an inhibitor the following reaction systems were used:-

(a) TB, EB, SB, ESB, from which a b_{30} value was derived,as before, for the enzyme-substrate reaction.

(b) TB, EB, ISB, EISB from which a b_{30} value was derived, for the enzyme-substrate reaction in the presence of a particular concentration [I] by the use of the equation:-

$$y\ (\mu l\ CO_2) = \text{Vol EISB} - \text{Vol EB} - \text{Vol ISB} + \text{Vol TB}.$$

The system ISB was included to check the extent of any volume change due to the combination of substrate and inhibitor preparations.

The EISB system measured the total enzyme catalysed reaction in the presence of inhibitor.

For the experiments the contents of the different reaction flasks were:-

TB		3.5 ml(B)		
EB	0.5 ml(E)	3.0 ml(B)		
SB		2.5 ml(B)	1.0 ml(S)	
ESB	0.5 ml(E)	2.0 ml(B)	1.0 ml(S)	
ISB		1.5 ml(B)	1.0 ml(S)	1.0 ml(I)
EISB	0.5 ml(E)	1.0 ml(B)	1.0 ml(S)	1.0 ml(I)

The effects of several concentrations of inhibitors were investigated simultaneously with a single enzyme-substrate reaction. Vf was 3500 µl.

There was no incubation of (E) and (I) prior to the reaction as (E) was mixed with (S) and (I) simultaneously.

By comparison of the inhibited reaction velocity with that of the uninhibited reaction the percentage inhibition produced by each concentration [I] was determined for each compound under investigation. These results are shown in Table 29. The % inhibition was plotted against log [I] for each compound (Fig 7) and from these graphs an idea of the molar concentrations giving 50% inhibition (I_{50} values) was gained (Table 29). In the case of physostigmine the I_{50} value was derived from a regression analysis applied to the results at the values [I] of 0.1, 0.5 and 1.0 µmol/l.

These initial results suggested that the two benzotriazinium compounds were weak inhibitors of AcChE compared with physostigmine, being of the order of 400 - 1,000 times less potent under the conditions of the test. The I_{50} value for physostigmine was within the range 10^{-6} - 10^{-8} mol/l quoted by Long (1963).

Table 29

Percentage inhibition of AcCh-AcChE reaction produced by three inhibitors (I) at 37°C and measured by Warburg Manometry.

[AcChE] - 0.05 units/ml [AcCh] - 2.0 m mol/l

I	[I] µmol/l							
Physostigmine Sulphate	0.01	0.1	0.5	1.0	5.0	10.0	I_{50}	p I_{50}
Mean % inhibition	-3.38	31.38	70.93	89.64	96.36	94.37	0.21 µmol/l	6.67
Standard error	6.24	3.18	2.20	2.33	5.25	1.49		
Number of determinations	2	4	2	4	2	2		
PMBI	0.286	2.86	28.6	286.0				
Mean % inhibition	1.22	-4.15	26.57	77.09			89 µmol/l	4.05
Standard error	0.44	0.97	8.88	5.25				
Number of determinations	2	2	2	2				
BnPBI	0.286	2.86	28.6	286.0				
Mean % inhibition	-6.04	-0.71	4.90	59.50			199.5 µmol/l	3.70
Standard error	8.45	3.41	8.90	5.51				
Number of determinations	2	2	2	2				

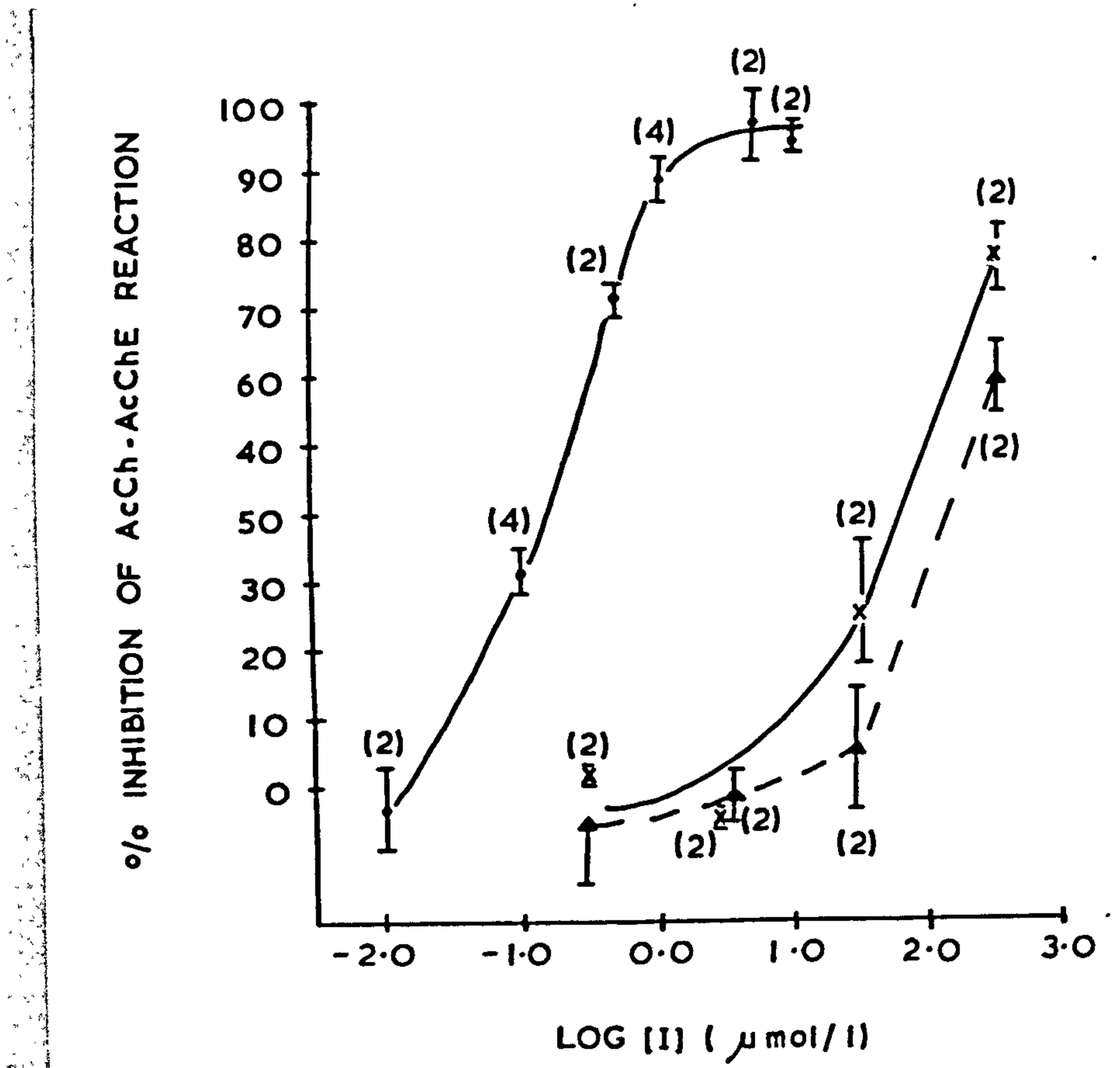


Fig 7.

Percentage inhibition of AcCh-AcChE reaction produced by three inhibitors (I) - physostigmine sulphate PMBI and BnPBI - at 37°C and measured by Warburg manometry.

General Discussion on results from the Warburg manometric determination of AcChE activity.

It has been shown that, as expected from a general consideration of enzyme reactions (Dixon and Webb, 1964), the magnitude of the reaction velocity (b_{30}) for the AcChE catalysed hydrolysis of AcCh was governed by the relative concentrations [E] and [S] utilised. Even in the twenty-four more comprehensive experiments with single, fixed, concentrations [E] and [S] the magnitude of the b_{30} values could vary significantly from day to day. Additionally these values were seen to have been significantly influenced by the contribution made to the volume changes by the combined effects of the enzyme preparation itself and the substrate preparation itself.

The latter results indicated the separate influences of enzyme and substrate preparations on the b_{30} values. The volume changes due to the enzyme preparation (0.05 units/ml) were shown in all but one of the twenty-four experiments to be negative and though of a generally low order to be subject to a high coefficient of variation. An awareness of volume changes due to the enzyme is important particularly in the case of crude enzyme preparations. Augustinsson (1948) indicated that when horse erythrocyte haemolysate was used as the source of AcChE carbonic anhydrase also present in the haemolysate reacted with the bicarbonate solution used by the Warburg method to liberate CO_2. The extent of this liberation was related to the enzyme concentration used. Although the liberation was essentially complete in two to three minutes from the mixing of enzyme and the bicarbonate its effect was such as to cause the graph relating the production of CO_2 to time to intercept the y axis at values greater than zero. In turn this created an error in the subsequent b_{30} value which had to be corrected. The need to correct Warburg results for the CO_2 evolved by washed red blood cells in the absence of AcCh was also noted by Witter (1962) in a study of the cholinesterase of rat blood. Mendel and Hawkins (1950)

pointed out that proteins may 'retain' acid and thus cause less CO_2 to be evolved than the amount of acid formed. They also pointed out that, by contrast, tissue homogenates, particularly liver, may themselves cause an appreciable evolution of CO_2 over a period of thirty minutes. Augustinsson (1948), however, showed that of many tissue preparations used as sources of cholinesterase most gave off no CO_2, or very small (1 to 3 μl) volumes, over thirty minutes. As these changes were claimed to be within the limits of error of the method he ignored them.

The influence of volume changes due to the substrate preparation was mentioned by Ammon (1933). He measured the spontaneous hydrolysis of AcCh chloride at 37.5°C and pH 7.4 and claimed that it could be disregarded. His subsequent results showed, however, that it may have comprised 10% or more of the liberated CO_2 volume at thirty minutes. In the present experiments such changes were small when [AcCh] was 2.0 m mol/l but much more variable than those due to the enzyme preparation and included both positive and negative changes. The average change in volume due to the substrate itself in the twenty-four experiments indicated that spontaneous hydrolysis was not a significant problem under the conditions of the test (pH 7.4, 37°C). This might be expected from the Warburg studies of Augustinsson (1948) on the non-enzymic hydrolysis of AcCh salts. These showed that of the three salts, chloride, bromide and iodide, the chloride was the most susceptible to spontaneous hydrolysis. At 37.5°C even this salt caused an evolution of CO_2 of less than 10 μl over thirty minutes at concentrations of 10 m mol/l or less. The mean change of -0.62 μl CO_2 in the twenty-four experiments with AcCh perchlorate (2.0 m mol/l) in the present experiments may thus be compared with the mean change of +4.0 μl CO_2 in thirty minutes produced by AcCh Chloride (1.10 m mol/l) reported by Augustinsson (1948). It was considered desirable to make such compensations during the calculations of reaction velocity. Otherwise a full separate investigation would have to be carried out

at all possible concentrations [E] and [S] proposed for study in order to ascertain not only the amount of compensation necessary but also whether or not this made a significant difference to the calculated reaction velocity. This may be the case with non-enzymic hydrolysis of substrate at low enzyme concentrations (Augustinsson, 1948).

The results of Augustinsson (1948) relating the non-enzymic hydrolysis of AcCh salts at 37.5°C to their concentrations were examined further. Over the concentration range 0.1 to 100 m mol/l these relationships were described by curves with the hydrolysis increasing markedly at concentrations above 10 m mol/l. At concentrations 0.10 to 10.0 m mol/l the spontaneous hydrolysis was less than 7.0 µl/30 minutes and appeared to be rectilinearly related to concentration. Regression analyses were performed on these latter results by the present author as they covered the range of substrate concentrations used in the present experiments. The analyses were of the regression on substrate concentration of the mean volumes of CO_2 liberated in thirty minutes in an unspecified number of three to six experiments. The substrate concentration ranges were 0.33 to 11.0 m mol/l for the chloride, 0.265 to 8.84 m mol/l for the bromide and 0.22 to 7.32 m mol/l for the iodide. The errors $\left(\frac{Sb}{b}\right)$ x 100% for the regression lines were 18.50% for the chloride, 18.51% for the bromide and 33.07% for the iodide showing the very approximate linear nature of this relationship.

The method of compensation adopted by Augustinsson (1948) was to subtract the mean volume of CO_2 liberated in thirty minutes by the appropriate concentration of substrate from the volume of CO_2 released at thirty minutes by enzyme catalysed hydrolysis. No indication of the precision with which the compensatory volume had been determined was given. This method of compensation was again advanced by Augustinsson in 1955b and 1957. Witter (1962) also corrected reaction velocities by subtraction of a fixed amount of CO_2 from the observed thirty minute value. This practice was defended (Witter, 1963) with the claim that

the correction was quite reproducible for a given batch of AcCh. The results of the present experiments were not in agreement with such a claim.

The implication in the work of Augustinsson (1948) was that the compensatory volume changes for spontaneous hydrolysis had not been determined at the same time as those due to enzyme catalysed hydrolysis. Mendel and Hawkins (1950) and Bockendahl and Ammon (1965), however, stated that controls for non-enzymatic substrate hydrolysis should always be included in the experiment.

Although the b_{30} values have been compensated for the volume changes induced by non-enzymatic mechanisms, the order in which the four manometers required to give each compensated b_{30} value were read appeared not to be of importance.

Just as certain factors have clearly influenced the magnitude of the b_{30} values so such factors may also have influenced the precision of these results. Here it was considered necessary to evaluate not only the variation amongst the b_{30} values but also the precision of the regression lines from which they were derived.

In any discussion of precision it is important to judge and state the criteria by which the precision of results might be deemed acceptable. It is further important to distinguish between acceptable levels of precision in routine experimental measurements and the 'best' level of precision seen in results only exceptionally. Acceptable levels of precision will vary according to factors involved in the assay being performed, e.g. the complexity of the assay technique, the opportunity for repetition or the method of deriving the results. Further, an acceptable precision amongst results may also vary according to the use to which the results are to be put.

Robinson (1971) quoting "Tonk's criteria" for desirable standards of analytical performance suggested that limits of error for any analysis should be $\pm \frac{1}{4}$ of the 'normal range' or $\pm$ 10% of the best

estimate of the true value, whichever was the lower. By "Tonk's criteria", the coefficient of variation for an acceptable assay should be less than 5%.

In a discussion of the estimates of kinetic parameters V_{MAX} and K_M in enzymology, Colquhoun (1969) considered that a coefficient of variation amongst reaction velocities of 4.6 per cent at a given substrate concentration was reasonable.

With regard to the manometric determination of cholinesterase activity it is difficult to gauge the acceptable level of precision which may be expected routinely. Augustinsson (1948), as evidence of the reliability of the technique, gave the mean result of eighteen determinations of activity of a cholinesterase preparation from Helix blood as 131.2 μl CO_2 with a standard deviation of 2.0, a coefficient of variation of less than 2%. In 1963 the same author simply stated that the precision of the method was high (2-3%) whilst in 1971 he stated that 'the accuracy can be within $\pm$ 2%'. Witter (1962) in a critical analysis of the use of Warburg manometry for determination of rat blood cholinesterase said that duplicate analyses usually checked within 1 or 2%. In 1963 Witter made the more equivocal statement that the precision and accuracy of the technique can be within $\pm$ 1%.

If the results of the present Warburg experiments are examined in the light of these criteria for precision it is found that the precision of results can be less than 1 or 2%. Table 10 shows four sets of b_{30} values with coefficients of variation of 2% or less. Even the earliest of the present experiments showed that the occasional set of results (Table 5) could have a coefficient of variation of less than 1%.

The results of the present Warburg experiments were also examined on the basis that a coefficient of variation of less than 5% in results from an assay was acceptable (Robinson, 1971). Table 5 showed that of nine sets of repeated b_{30} measurements at different

concentrations [E] and [S] only one set had a coefficient of variation of less than 5%. In Table 7 none of the five sets of results had less than a 5% coefficient of variation. By contrast seven of the eighteen sets of results in Table10 showed a coefficient of less than 5%. These were results obtained after refinement of the experimental technique and adjustment of the timing of manometric readings.

In the later Warburg experiments at single, fixed concentrations [E] and [S] the coefficient was only less than 5% on one of the eight days, although it was 10% or less on seven of the days. It was shown in these experiments that the carrying out of the experiments on different days had, as might be expected, an adverse effect on the precision of the b_{30} values.

Previous discussion indicated that in some experiments the accuracy of the b_{30} values had been shown to be significantly affected by compensation for volume changes due to the enzyme and substrate preparations. It was also noted that the precision of these compensations was very low and was probably only tolerable because of the small numerical value of the compensation compared with the corresponding volume change due to the enzyme catalysed hydrolysis. It might be considered that this low precision was a factor favouring the application of compensation in each experiment, as in this research, rather than subtracting a fixed volume of CO_2 from the uncorrected reaction velocities as did Augustinsson (1948,1957) and Witter (1962, 1963). Whilst the evidence from these present experiments was that the compensation had significantly altered the accuracy but not the precision of the results there may well be experimental situations in which precision was also influenced by the compensation. This possibility would also be more conveniently allowed for by a compensation built into the experiments than by a separate investigation.

The results of the present research have indicated that the precision of the regression lines from which the b_{30} values were

derived varied with the concentrations [E] and [S] and with the frequency and duration of manometric readings. Myers (1952) clearly demonstrated, with AcCh as substrate, that the reaction time for cholinesterases was limited not only by the concentrations of AcCh but also by the total amount present in the reaction system. He emphasied the need to reduce the time intervals between manometric readings at low substrate concentrations, particularly when small volumes of solutions were employed. Reference to Table 9 showed that after making adjustment in the timing of manometric readings to yield an adequate number of points for regression analysis there were certain concentrations [E] and [S] at which more precise analyses were possible than others. The chosen expression for error $\left(\frac{Sb}{b}\right)$ x 100% may be regarded as a coefficient of variation for the slope of the regression line and a coefficient of less than 5% regarded as reasonable. It was found that most of the lines with such coefficients were at the higher concentrations of both enzyme and substrate investigated (0.05 and 0.10 units/ml and 2.0 to 4.0 m mol/l respectively). As stated this finding influenced the choice of the single fixed concentrations [E] and [S] for the later more comprehensive experiments. The choice was apparently justified in that the errors were less than 5% in nineteen of the twenty-four experiments (Table 16). When the reaction velocities for these experiments were recalculated using the simpler mathematical expression the errors were less than 5% on twenty of the twenty-four occasions (Table 22).

The errors of the regression lines might be used as a very rough indication of the improvement in the use of the Warburg technique during the current research. Results may be thus compared from experiments using the concentrations [E] and [S] of 0.05 units/ml and 2.0 m mol/l respectively. In the earliest experiments (Table 4) none of the four regression lines at these concentrations had a coefficient of less than 5%. In the experiments shown in Table 9 the

two coefficients were 3.18 and 6.26% whilst in the latter experiments, as stated, nineteen out of twemty-four coefficients had values less than 5%

It was considered that the b_{30} value might be less convenient, as a measure of reaction velocity, than the slope of the corresponding regression line in that its calculation followed from the determination of the slope. For example the b_{30} value might have required correction for error existing when the line did not cross the ordinate expressing CO_2 production at the origin. Determination of the value might also have required extrapolation beyond the range of observations when the reaction rate ceased to be linear before thirty minutes.

Most Warburg manometric determinations of cholinesterase have in fact involved expression of the results in terms of the amount of CO_2 produced over a given period of time. Ammon (1933) determined CO_2 production as cc/hr. Augustinsson (e.g. 1948,1955, 1957) used the b_{30} value as did Smallman and Wolfe (1954). Myers (1952) gave examples of experiments in which the results were expressed in μl CO_2/20 min. whilst Mendel and Hawkins (1950) stated that results might be expressed as the μl CO_2 evolved or μmoles AcCh hydrolysed in twenty, thirty or sixty minutes. Witter (1962) also calculated the μmoles CO_2 produced in thirty minutes. In many of these cases the activities were, in turn, related to weight of tissue or volume of blood preparation used as the enzyme source.

If a large number of experiments were required in experimental conditions where it was known that the production of CO_2 bore a rectilinear relation to time over thirty minutes and if it was known that no correction was required in respect of the intercept error then an observed CO_2 value at thirty minutes would be a convenient measure of reaction velocity. When this was not the case the slopes of the regression lines, which show the same variations as the corresponding b_{30} values, might be preferred. However, the results of the present

research indicated that, under certain conditions, the ratios of the errors of the b_{30} values to the b_{30} values were consistently and significantly less than the corresponding ratios for slope. This appeared to be a factor clearly favouring the use of the b_{30} value under these conditions.

With regard to the chosen experimental conditions the following points emerged from the present research.

The optimum concentration of AcCh as substrate for AcChE appeared to be approximately 4.0 m mol/l. This compared with quoted values of 3.0 m mol/l (Augustinsson, 1949) and 2.5 to 3.0 m mol/l (Cohen and Oosterbaan, 1963). Nachmansohn and Wilson (1955) stated that the optimum substrate concentration varied with the source of AcChE, being 4.0 m mol/l for electric tissue and 7.0 m mol/l for erythrocyte enzyme. Further the present work showed that the lowest chosen concentration of substrate which might be reasonably detected was approximately 0.5 m mol/l. At the lower enzyme concentrations not enough CO_2 was liberated at this substrate concentration for a precise determination of the b_{30} value. At the highest concentrations $[E]$ the initial reaction was complete in a short period of time, again raising problems of accuracy of determination. This finding confirmed earlier reports. Barlow (1964) stated that the lowest concentration of substrate detectable was 0.2 m mol/l whilst Witter (1963) and Augustinsson (1971) quoted a minimum detectable substrate concentration of 0.4 m mol/l for the Warburg method.

With regard to the master enzyme solutions (M.E.S) the results in Tables 16and 17 suggested that, as formulated and stored, the M.E.S were stable for at least a fortnight. The results in Table 17 were obtained over fourteen days, the mean b_{30} values tending to vary in a random manner over that period. Had the M.E.S been deteriorating a steady reduction in the mean b_{30} value might have been found. There is a body of evidence regarding the stability of cholinesterase

enzymes. Nachmansohn and Wilson (1955) stated that AcChE of electric tissues had great atability and could be kept in the refrigerator for years without loss of activity and that freezing did not affect the enzyme. Augustinsson (1963) pointed out that partly purified preparations of cholinesterase could be stabilized by the addition of certain albumins or gelatin. Witter (1962) claimed that the enzymes in the plasma or red cells of rat blood were stable and could be stored at 5°C for as long as five days, plasma being capable of storage in the frozen state without a drop in activity for several months. In 1963 Witter stated that separated plasma and red cell cholinesterases were stable for several weeks if kept at 0 to 5°C. The stabilising effect of gelatin on γ cholinesterase was, in turn, demonstrated by Beckett, Vaughan and Mitchard (1969). Such reports formed the basis for the inclusion of gelatin in the M.E.S in the present research.

A potential problem with the technique adopted was that each determination of reaction velocity involved measurements in four separate reaction systems each of which contributed to the overall error of the measurement. This snag has to be set against the fact that the method allows several determinations to be run simultaneously without alterations to the volume of reaction medium during the course of the experiment (Mendel and Hawkins, 1950).

It was considered that the investigation of the Warburg method had been sufficient to indicate the probable accuracy and precision with which reaction velocities might be measured under given experimental conditions. Also an initial application of the method to studies of cholinesterase inhibitors had been essayed. An investigation of the use of the pH - stat method for the determination of reaction velocity appeared to be appropriate at this stage. With instrumentation for both methods available it was considered that one of the methods might prove superior, as a method for the determination of kinetic parameters in connection with the assessment of cholinesterase inhibitors. The

need to investigate the alternative method was also prompted by the claim that the use of the Warburg method could present difficulties in the assay of reversible esterase inhibitors (Augustinsson, 1971).

The decision to investigate the pH - stat method was made in the knowledge that certain aspects of the Warburg research would have benefited from more intensive study. In particular the investigation of the relative merits of the b_{30} value and the slope of the corresponding regression line as measures of reaction velocity was considered to merit future investigation and analysis under a greater variety of experimental conditions.

Appendix

Determination of reaction velocities, K_M and V_{MAX} for the interaction between AcCh and AcChE at 37°C.

Experiment 2.63

Substrate (S) AcCh perchlorate. Master solution (10 m mol/l) made in deionised water. Dilutions prepared in 'gassed' reaction medium (B)

Enzyme (E) AcChE from bovine erythrocytes. Master solution 1.25 units/ml. Diluted with 'gassed' (B) to give a final flask concentration (F.F.C.) of0.10 units/ml.

Reaction medium (B) NaCl 0.15 mol/l; $MgCl_2$ 0.04 mol/l; $NaHCO_3$ 0.034 mol/l

Gas 5% CO_2/95% N_2

Reaction System		Flask constant k.	Contained			F.F.C(s) m mol/l
TB	Thermobarometer	1.35	2.5 ml (B)			
EB	Enzyme-buffer 'blank'	1.31	0.5ml(E)	2.0 ml(B)		
S_1B	Substrate [S_1]-buffer 'blank'	1.32		1.5 ml(B)	1.0 ml(S)	4.0
ES_1B	Enzyme catalysed hydrolysis at [S_1]	1.25	0.5ml(E)	1.0 ml(B)	1.0 ml(S)	4.0
S_2B	Systems corresponding with S_1B and ES_1B above.	1.22		1.5 ml(B)	1.0 ml(S)	3.0
ES_2B		1.27	0.5ml(E)	1.0 ml(B)	1.0 ml(S)	3.0
S_3B		1.28		1.5 ml(B)	1.0 ml(S)	2.0
ES_3B		1.40	0.5ml(E)	1.0 ml(B)	1.0 ml(S)	2.0
S_4B		1.19		1.5 ml(B)	1.0 ml(S)	1.0
ES_4B		1.23	0.5ml(E)	1.0 ml(B)	1.0 ml(S)	1.0
S_5B		1.20		1.5 ml(B)	1.0 ml(S)	0.5
ES_5B		1.32	0.5ml(E)	1.0 ml(B)	1.0 ml(S)	0.5

For each reaction system the following manometer readings (h mm) were taken at the stated times (Table 30).

For each system the manometer readings (h mm) were multiplied by the appropriate flask constants (k) to give the volume changes Vol (μl) shown in Table 31.

For each substrate concentration [S] the volume of CO_2 (μl) at any stated time was derived from the equation:-

y (μl CO_2) = Vol ESB - Vol EB - Vol SB + Vol TB (Table 32).

For each concentration [S] a graph of y against time was plotted (Fig.8) For each graph the points which lay on a straight line were judged by eye. The best straight line was calculated for these points by regression analysis (Table 32) and b_{30} values calculated.

From Fig.8 it was judged that the values y were linearly related to time for the following periods:-

[S] m mol/l	time (mins) for which y is linear.
4.0	0 - 40.0
3.0	0 - 40.0
2.0	0 - 25.0
1.0	0 - 17.5
0.5	0 - 10.0

For the derivation of K_M and V_{MAX} a graph of $\frac{1}{b_{30}}$ against $\left[\frac{1}{s}\right]$ was plotted (Fig. 8). The best straight line was calculated for the points by regression analysis.

The intercept of the regression line with the ordinate $\left(\frac{1}{V_{MAX}}\right)$ was used for the derivation of V_{MAX}.

The intercept of the regression line with the abcissa $\left(-\frac{1}{K_M}\right)$ was used for the derivation of K_M.

The regression analysis for the line shown in Fig. 8 gave:-

b	Sb	c(intercept at ordinate)	intercept at abcissa
0.0048	0.0006	0.0090	-1.875

From these results K_M was 0.53 m mol/l

V_{MAX} was 111.11 μl CO_2/30 min.

Table 30 - Manometer readings (h mm) in reaction systems.

System	Flask constant k		Time (mins) 2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	22.5	25.0	27.5	30.0	32.5	35.0	37.5	40.0
TB	1.35	h mm reading	2	3	4	4	5	3	5	3	4	5	5	5	5	4	4	5
EB	1.31		7	8	8	8	10	9	10	9	11	11	11	11	11	9	9	9
S_1B	1.32			4		5		6		6		7		8		5		5
ES_1B	1.25			19		35		50		64		81		89		102		113
S_2B	1.22			8		12		14		15		17		17		15		15
ES_2B	1.27			17		35		49		65		83		94		102		110
S_3B	1.28			5		9		9		9		9		9		8		8
ES_3B	1.40			22		37		51		60		70		74		75		77
S_4B	1.19		1	2	2	3	4	3	3	4	5	5	5	4	5	2	2	2
ES_4B	1.23		13	19	24	32	35	39	42	44	45	46	46	46	46	44	44	44
S_5B	1.20		5	6	7	10	9	8	8	9	9	9	8	8	7	7	7	7
ES_5B	1.32		10	16	19	24	24	24	25	25	25	26	25	25	24	23	23	23

Table 31
Volume changes (μl) in reaction systems.

System	Time (mins.)															
	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	22.5	25.0	27.5	30.0	32.5	35.0	37.5	40.0
TB	2.70	4.05	5.40	5.40	6.75	4.05	6.75	4.05	5.40	6.75	6.75	6.75	6.75	5.40	5.40	6.75
EB	9.17	10.48	10.48	10.48	13.10	11.79	13.10	11.79	14.41	14.41	14.41	14.41	14.41	11.79	11.79	11.79
S_1B		5.28		6.60		7.92		7.92		9.24		10.56		6.60		6.60
ES_1B		23.75		43.75		62.50		80.00		101.25		111.25		127.50		141.25
S_2B		9.76		14.64		17.08		18.30		20.74		20.74		18.30		18.30
ES_2B		21.59		44.45		62.23		82.55		105.41		119.38		129.54		139.70
S_3B		6.40		11.52		11.52		11.52		11.52		11.52		10.24		10.24
ES_3B		30.80		51.80		71.40		84.00		98.00		103.60		105.00		107.80
S_4B	1.19	2.38	2.38	3.57	4.76	3.57	3.57	4.76	5,95	5.95	5.95	4.76	5.95	2.38	2.38	2.38
ES_4B	15.99	23.37	29.52	39.36	43.05	47.97	51.66	54.12	55.35	56.58	56.58	56.58	56.58	54.12	54.12	54.12
S_5B	6.00	7.20	8.40	12.00	10.80	9.60	9.60	10.80	10.80	10.80	9.60	9.60	8.40	8.40	8.40	8.40
ES_2B	13.20	21.12	25.08	31.68	31.68	31.68	33.00	33.00	33.00	34.32	33.00	33.00	31.68	30.36	30.36	30.36

Table 32 - Volumes CO_2 (y µl) given by concentrations [S].

[S] m mol/l	Time (mins.) 2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	22.5	25.0	27.5	30.0	32.5	35.0	37.5	40.0
4.0		12.04		32.07		46.84		64.34		84.35		93.03		114.51		129.61
3.0		5.40		24.73		37.41		56.51		63.51		90.98		104.85		116.36
2.0		17.97		35.20		52.14		64.74		78.82		84.42		88.37		92.52
1.0	8.33	14.56	22.06	30.71	31.94	36.66	41.74	41.62	40.39	42.97	42.97	44.16	42.97	45.35	45.35	46.70
0.5	0.73	7.49	11.60	14.60	14.53	14.34	17.05	14.46	13.19	15.86	15.74	15.74	15.62	15.57	15.57	16.92

Regression analyses of y on time applied for results over the periods stated in text.

[S]m mol/l	$\frac{1}{[S]}$	b	Sb	c(y intercept)	y at 30 mins	b_{30}value	$\frac{1}{b_{30}}$
4.0	0.25	3.3185	0.0781	-2.57	96.99	99.56	0.0100
3.0	0.33	3.2024	0.1200	-9.59	86.49	96.08	0.0104
2.0	0.50	3.0248	0.1140	4.40	95.14	90.74	0.0110
1.0	1.00	2.2044	0.1682	4.53	70.66	66.13	0.0151
0.5	2.00	1.8288	0.2416	-2.83	52.04	54.87	0.0182

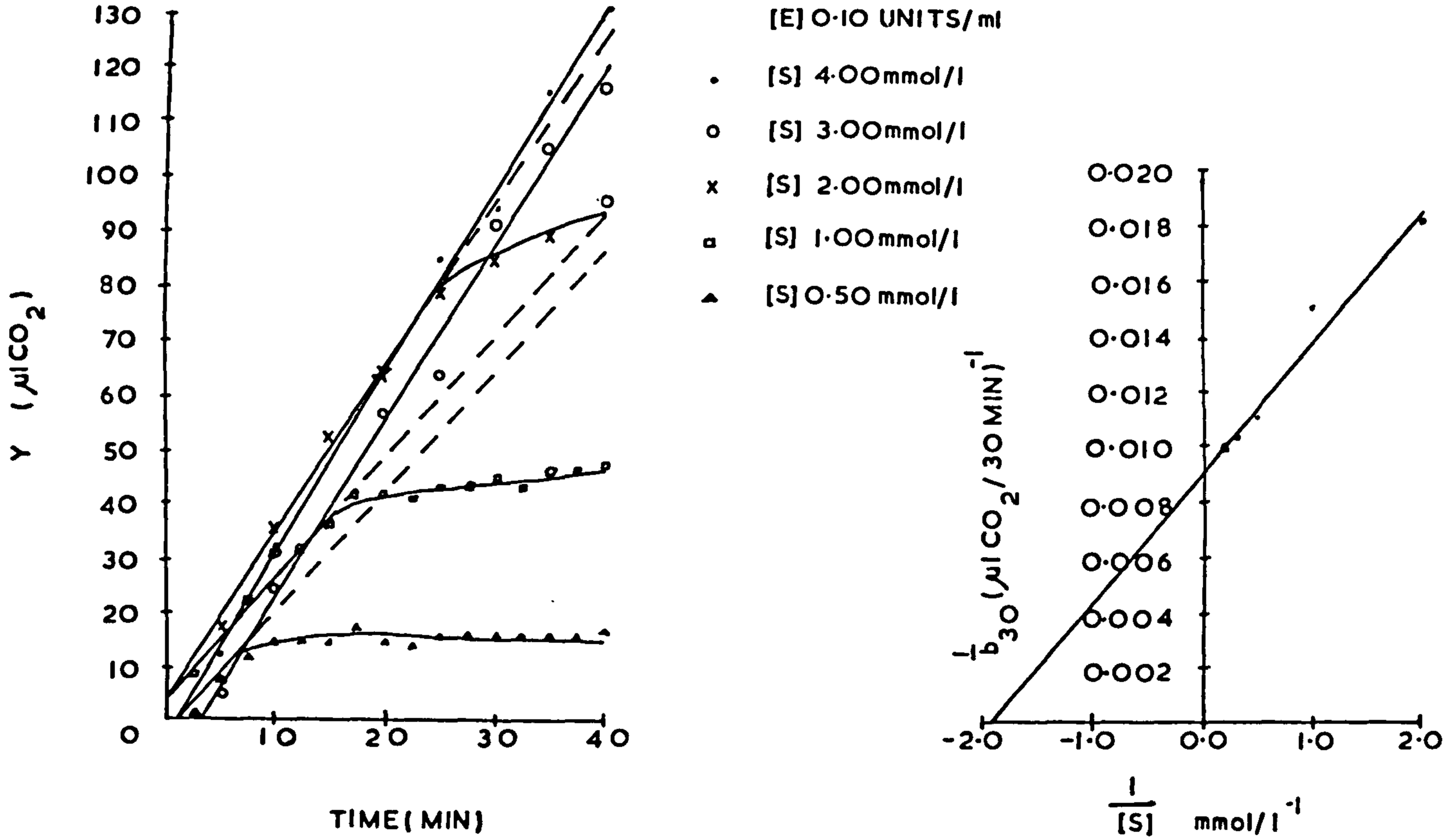


Fig 8.

Graphs illustrating the derivation of b_{30} values, K_M and V_{MAX} for the AcCh-AcChE reaction at 37°C.

EXPERIMENTS USING THE pH-STAT METHOD TO DETERMINE ACETYLCHOLINESTERASE ACTIVITY.

<u>An investigation into the use of the pH - stat method for the determination of acetylcholinesterase activity.</u>

The pH - stat method was applied to a study of the reaction between AcChE and AcCh. This reaction was studied on various occasions at 37°C, at pH 7.0 and pH 7.4 at various concentrations [E] and [S], and in the presence of inhibitors. Reaction velocities were determined from the volumes of 0.02N NaOH titrant required to neutralise the acid released during the reaction over a given period of time. The reaction velocities were expressed as the number of micromoles of AcCh hydrolysed per minute.

<u>Apparatus.</u>

The apparatus is shown in Fig 9 . It comprised a titration assembly (Type TTA3, Radiometer) used in conjunction with an automatic titrator (Type TTT2b, Radiometer), an autoburette (Type ABU 12b, Radiometer) and a titrigraph (Type SBR3, Radiometer).

The titration assembly comprised a reaction vessel (50 ml capacity) for the required reagents. A water jacket, through which was circulated water at 37°C, enclosed the vessel. An electrode head supported glass and reference pH electrodes and also a stirrer all of which dipped into the reaction mixture. The stirrer was driven by an electric motor. The electrode head also had two apertures through which were passed a titrant delivery tube and a tube delivering nitrogen to provide an inert atmosphere for the reaction. Additionally a third aperture, normally closed by a stopper, allowed addition of substrate or inhibitor to the vessel during the course of the experiment.

The pH electrodes were connected to the automatic titrator which served both as a meter to monitor the pH of the reaction and also as a control for the delivery of titrant to the reaction vessel. Buffer and temperature controls were provided on the pH meter. Adjustment of an end point control allowed the pH at which a reaction took place to

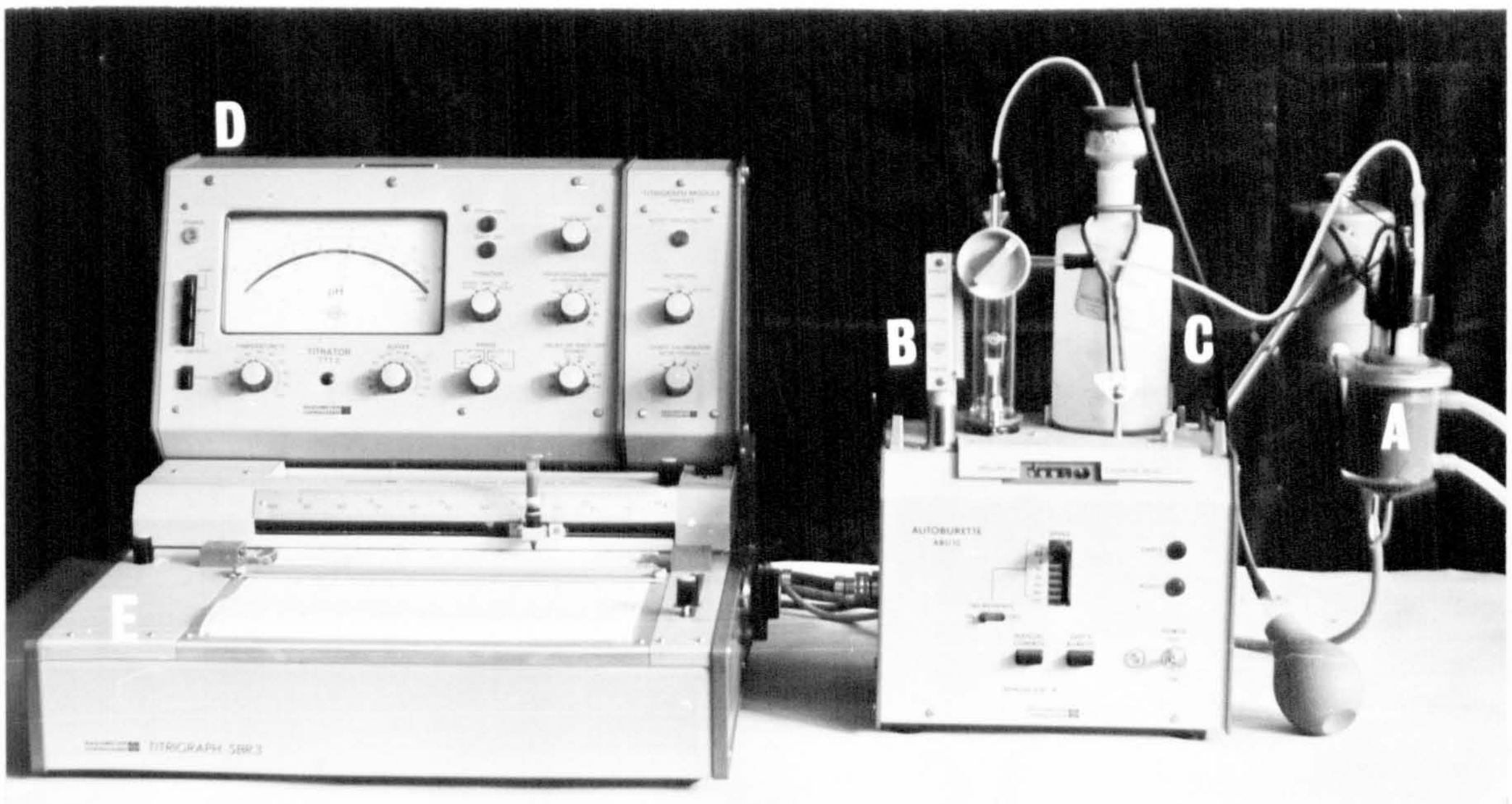


Fig 9.

Apparatus used for pH - stat experiments.

A - water jacketed reaction vessel.

B - Autoburette.

C - Reservoir for titrant.

D - Automatic titrator.

E - Titrigraph recorder.

be preset. A fall in pH below the end point, resulting from acid production during the reaction, caused the titrator to activate the autoburette from which titrant was delivered to the reaction vessel. The pH range about the end point over which the titrator controlled titrant delivery could be varied. Over this range the titrant addition was incremental, smaller increments at longer time intervals being added as the pH neared the end point. The titrator was started and stopped by the use of push buttons.

The autoburette was a motor-driven piston burette of 2.5 ml capacity. Controls permitted automatic titration, refilling of burette with titrant from a reservoir and the expulsion of air from the burette. The reservoir was fitted with an absorption head filled with soda lime to absorb atmospheric CO_2. As an alternative to being controlled from the titrator the burette could be operated manually at a variety of speeds. The burette piston was mechanically coupled both to a digital counter and, via a flexible drive shaft, to the titrigraph recorder. The counter showed the volume of titrant added to the third decimal place and could be reset by push button.

The titrigraph could be run at various speeds and could be adjusted so that full pen movement corresponded to either 1.0 ml or 2.5 ml of titrant delivered.

Solutions.

Reaction medium (RM) contained NaCl 0.15 mol/l and $MgCl_2$ 0.04 mol/l in deionised water. The salts were kept as stock solutions (1.5 and 0.4 mol/l respectively) prepared in one litre batches. The strengths of the solutions were checked by the use of a chloride meter.

Enzyme solutions (E) contained the AcChE from bovine erythrocytes (Sigma Chemical Co) described for the Warburg manometric experiments. Master enzyme solutions (M.E.S), 1.25 units/ml, were prepared in RM containing 1% partially hydrolysed gelatin.

A few experiments were performed utilising AcChE from electric

eel. This enzyme was obtained commercially (Sigma Chemical Co.) as a lyophilised salt-free powder in 2000 unit quantities. A.M.E.S was prepared as with the erythrocyte enzyme but containing 25 units/ml. This was diluted prior to use with RM.

Unless otherwise stated it will be assumed that references to enzyme relate to erythrocyte enzyme.

Substrate solution (S) contained AcCh perchlorate (B.D.H - Biochemical Grade) dissolved in RM.

All solutions were stored at $4^{o}C$ in the dark between experiments.

Substrate solutions were prepared freshly prior to each day's experiments.

Titrant was 0.02 N NaOH prepared by diluting a standardised normal volumetric solution of NaOH (B.D.H.) 1 in 50 with deionised water. The titrant normality was, in turn, checked against standard volumetric hydrochloric acid (B.D.H.) using phenolpthalein as indicator.

Nitrogen The nitrogen used in the experiments was obtained commercially from the British Oxygen Co.Ltd.

Reaction mixtures The total volume of reaction mixture was 25.0 ml in all experiments.

For enzyme-substrate studies this comprised 23.0 ml (RM)and 1.0 ml (E) and 1.0 ml (S) of appropriate concentration.

For enzyme-substrate-inhibitor studies 1.0 ml of inhibitor solution (I) of appropriate concentration was also added with a consequent reduction in the volume (RM) to 22.0 ml.

Experimental Protocol

At the start of each day's experiments the reaction vessel was heated to $37^{o}C$ and the pH meter of the titrator was calibrated with a buffer solution at this temperature. The burette was filled with titrant and the burette and titrant delivery tube cleared of any entrapped air. The working of the recorder pen was checked and the required end-point pH was preset on the titrator.

Buffer solution in the reaction vessel was replaced by the appropriate volumes of (RM) and (E). At this point and for the remainder of each experiment nitrogen was blown over the surface of the reaction mixture to exclude CO_2. The mixture of (RM) and (E) was allowed to equilibrate for temperature for ten minutes whilst being stirred.

After ten minutes the contents of the reaction vessel were then adjusted to the preset end-point. This was achieved by starting the titration at a speed of 0.125 ml titrant/min, the titration being controlled when the pH of the vessel contents was within 0.5 pH unit of the end point. During this pH adjustment addition of titrant was not recorded. At this stage just prior to the reaction the burette speed was changed to 0.25 ml/min. The pH range for the control of titrant addition was switched to within 0.05 pH units of the end-point.

For enzyme-substrate studies the recorder pen was then switched on and the digital counter of the autoburette reset. Recording proceeded for four minutes to check for any non-specific acid liberation from the enzyme preparation. Substrate solution was then added to start the reaction and the recording of titrant addition proceeded for six minutes. During this period the volumes of titrant added, as shown on the digital counter, were noted at various times. After six minutes the titration was stopped, the reaction vessel was emptied, washed with deionised water and dried in a stream of air. The pH electrodes, stirrer, titrant and nitrogen delivery tubes were also washed with deionised water and dried. The reaction vessel was then reloaded for the next experiment.

Some experiments were performed to check for spontaneous hydrolysis of substrate. Here the volume of (RM) was 24.0 ml and (E) was omitted.

For enzyme-substrate-inhibitor studies the mixture of (RM) and (E) was allowed to equilibrate and the pH adjusted to the desired end-point

as before. Any non-specific acid release due to the enzyme preparation was checked for one minute. (I) was then added and allowed to incubate with (E) for a predetermined time. During this time any acid release due to (I) could be detected. Substrate was then added and the reaction recorded for six minutes.

Calculation of reaction velocities.

A variety of methods is reported in the literature for calculating reaction velocities (v) from pH - stat experiments on cholinesterase. Similarly the duration of such experiments has varied. For example Witter (1963) stated that as little as two minutes was required to produce a rate curve for calculation of reaction velocity. Nabb and Whitfield (1967) titrated reactions for three minutes or until at least three minutes of the recording was linear. Their results were then calculated from deflections of the recorder during this period. Robinson and Robinson (1968) calculated results obtained during the second and third minutes of reaction from the slope of the recording of the volume of titrant added against time. Augustinsson (1971) stated that recording should proceed until it was linear for at least three minutes and that the reaction rate was read from the slope of the straight line recorded by the titrigraph. He also quoted methods employing automatic recording titrators in which the reaction times had varied between one and ten minutes.

These reported variations in measuring reaction velocities were considered together with the facility of the apparatus used in the present research to give both a graphical representation and a digital read-out of the volume of titrant added with time. It was decided that an initial investigation should be made of the derivation of reaction velocities and kinetic constants from the AcCh-AcChE reaction. In these experiments the use of the titrigraph curve and the digital counter for the derivation of results were compared over a given period of time. The results from the use of the counter were compared when readings were taken over five different periods of time.

Six experiments were performed at $37^{\circ}C$ and pH 7.0 each utilising a concentration [E] of 0.05 units/ml F.F.C and four concentrations [S] of 0.25, 0.5, 1.0 and 2.0 m mol/l F.F.C. The choice of [E] was vindicated by the subsequent characterisation of the enzyme system.

The order in which the substrate concentrations were studied was varied as shown:-

Experiment	Order of study of substrate concentrations (m mol/l)			
1	2.00	1.00	0.50	0.25
2	0.50	2.00	0.25	1.00
3	0.25	0.50	1.00	2.00
4	1.00	0.25	2.00	0.50
5	0.50	2.00	0.25	1.00
6	1.00	0.25	2.00	0.50

For each reaction the reaction velocities were calculated in six different ways:-

1. From the tangent to the recorded titrigraph curve during the second minute of the reaction.

2. From the digital counter read-out of titrant added during the second minute of the reaction.

3. From the digital counter read-out of titrant added during the third minute of the reaction.

4. From the digital counter read-out of titrant added during the third and fourth minutes of the reaction.

5. From the digital counter read-out of titrant added during the third, fourth and fifth minutes of the reaction.

6. From the digital counter read-out of titrant added during third to the sixth minutes of the reaction inclusive.

From the values obtained in these six different ways the number of ml. of 0.02N NaOH used per minute and thus the number of μmoles AcCh hydrolysed per minute were determined for each substrate concentration. V_{MAX} and K_M were also derived by application of regression analyses to both the Lineweaver - Burk plot ($\frac{1}{v}$ on $\frac{1}{[S]}$) and the Hofstee plot (v on $\frac{v}{[S]}$) as it was considered that both these plots might be applied to results from subsequent experiments.

Results and discussion

Table 33 shows the mean reaction velocities, with standard errors obtained at different substrate concentrations and the mean kinetic parameters with standard errors. No results were calculated from the first minute of the reaction as it was considered that such results might be subject to inaccuracy due to the time required for adequate mixing of substrate with the other reagents and the slight, but finite, delay before the reaction was measurable. Additionally there was sometimes a very slight overshoot of the end-point during pH adjustment which again caused slight inaccuracy in measurement of reaction velocity during the first minute. pH errors in the first moments of titration were mentioned by Jensen-Holm et al (1959) and by Jorgensen (1959).

't' tests were performed between the mean reaction velocities derived by the six different ways for each of the four substrate concentrations. 't' tests were also performed between the mean kinetic parameters obtained by these ways and using both graphical plots. The results of these tests are shown in Table 34 .

Thirteen of the fifteen 't' tests at the 0.25 m mol/l concentration of AcCh showed significant differences though no differences were seen in the mean results obtained during the second minute by the use of the titrigraph curve or the digital counter read-out.

At the 0.5 and 1.0 m mol/l concentrations of AcCh only five and three 't' tests respectively showed significant differences, again with no significant difference in the results obtained during the second minute.

When [AcCh] , 2.0 m mol/l, was used only five of the 't' tests showed significant differences. Here, however, all the velocities calculated from the digital counter read-out during the second minute were significantly greater than the other velocities.

Table 33

Reaction velocities and kinetic parameters determined for AcCh-AcChE reactions at pH 7.0 and 37°C by the pH-stat method.

Reaction velocities expressed as μmoles AcCh hydrolysed per minute, K_M as m mol/l. [AcChE] =0.05 units/ml.

Each value is the mean of six determinations. cv = coefficient of variation%.se = standard error of mean

[AcCh] m mol/l	Plot			Results derived from tangent to the titrigraph curve during the second minute.	Results derived from read-out of volume of titrant added during second minute	third minute	third & fourth minute	third to fifth minute	third to sixth minute.
0.25		v	mean	0.6769	0.7133	0.6100	0.5850	0.5378	0.5067
			se	0.0100	0.0375	0.0144	0.0138	0.0059	0.0046
			cv	3.61	12.87	5.77	5.80	2.68	2.22
0.50		v	mean	0.8902	0.9100	0.9067	0.8683	0.8411	0.8225
			se	0.0197	0.0300	0.0256	0.0145	0.0123	0.0104
			cv	5.41	8.08	6.93	4.08	3.59	3.09
1.0		v	mean	1.1398	1.1867	1.1300	1.1117	1.0955	1.0817
			se	0.0234	0.0272	0.0277	0.0111	0.0162	0.0157
			cv	5.03	5.61	6.00	2.44	3.62	3.56
2.0		v	mean	1.2117	1.3200	1.2000	1.2017	1.1911	1.1925
			se	0.0107	0.0369	0.0000	0.0105	0.0106	0.0087
			cv	2.17	6.84	0.00	2.13	2.17	1.79
	$v, \frac{v}{[S]}$	K_M	mean	0.2662	0.2952	0.3220	0.3573	0.4192	0.4697
			se	0.0117	0.0428	0.0230	0.0144	0.0167	0.0141
			cv	10.77	35.53	17.49	9.86	9.76	7.36
	$v, \frac{v}{[S]}$	V_{MAX}	mean	1.3937	1.5055	1.4413	1.4580	1.4949	1.5288
			se	0.0176	0.0575	0.0275	0.0176	0.0239	0.0210
			cv	3.10	9.36	4.67	2.96	3.91	3.37
	$\frac{1}{v}\frac{1}{[S]}$	K_M	mean	0.2677	0.2897	0.3561	0.3895	0.4676	0.5389
			se	0.0104	0.0415	0.0245	0.0264	0.0162	0.0154
			cv	9.49	35.06	16.83	16.63	8.51	6.99
	$\frac{1}{v}\frac{1}{[S]}$	V_{MAX}	mean	1.3941	1.4932	1.4906	1.5040	1.5624	1.6230
			se	0.0168	0.0556	0.0297	0.0335	0.0229	0.0240
			cv	2.96	9.12	4.88	5.46	3.59	3.62
			column	1	2	3	4	5	6

Table 34

't' tests on mean reaction velocities (v) and kinetic parameters (K_M, V_{MAX}) derived from the reaction between AcCh and AcChE at pH 7.0 and 37°C and shown in colums 1-6 Table 33

10 degrees of freedom in all tests. t (theoretical) P = 0.95, 10df = 2.23

t (calc) - calculated value of t.. D.S. - Difference Significant?.

t test between columns in Table	t tests on mean values, v, when [AcCh] (m mol/l) was								t tests on mean kinetic parameter values							
	0.25		0.50		1.00		2.00		K_M (v, $\frac{v}{[S]}$ plot)		V_{MAX} (v, $\frac{v}{[S]}$ plot)		K_M ($\frac{1}{v}$, $\frac{1}{[S]}$ plot)		V_{MAX} ($\frac{1}{v}$, $\frac{1}{[S]}$ plot)	
	t(calc)	D.S.	t(calc)	D.S.	t(calc)	D.S.	t(calc)	D.S.	t(calc)	D.S.	t(calc)	D.S.	t(calc)	D.S.	t(calc)	D.S.
1 & 2	0.94	No	0.55	No	1.31	No	2.82	Yes	0.65	No	1.86	No	0.52	No	1.71	No
1 & 3	3.82	Yes	0.51	No	0.27	No	1.09	No	2.16	No	1.46	No	3.33	Yes	2.82	Yes
1 & 4	5.38	Yes	0.90	No	1.09	No	0.67	No	4.92	Yes	2.58	Yes	4.29	Yes	2.93	Yes
1 & 5	12.00	Yes	2.11	No	1.56	No	1.37	No	7.50	Yes	3.41	Yes	10.37	Yes	5.92	Yes
1 & 6	15.48	Yes	3.04	Yes	2.06	No	1.39	No	11.11	Yes	4.93	Yes	14.62	Yes	7.81	Yes
2 & 3	2.57	Yes	0.08	No	1.46	No	3.25	Yes	0.55	No	1.01	No	1.38	No	0.04	No
2 & 4	3.21	Yes	1.25	No	2.56	Yes	3.09	Yes	1.37	No	0.79	No	2.03	No	0.17	No
2 & 5	4.63	Yes	2.12	No	2.88	Yes	3.36	Yes	2.70	Yes	0.17	No	3.99	Yes	1.15	No
2 & 6	5.47	Yes	2.76	Yes	3.34	Yes	3.36	Yes	3.87	Yes	0.38	No	5.63	Yes	2.14	No
3 & 4	1.25	No	1.30	No	0.61	No	0.16	No	1.30	No	0.51	No	0.93	No	0.30	No
3 & 5	4.65	Yes	2.30	Yes	1.07	No	0.84	No	3.42	Yes	1.47	No	3.80	Yes	1.92	No
3 & 6	6.85	Yes	3.04	Yes	1.52	No	0.86	No	5.48	Yes	2.53	Yes	6.33	Yes	3.47	Yes
4 & 5	3.14	Yes	1.43	No	0.82	No	0.71	No	2.81	Yes	1.24	No	2.52	Yes	1.44	No
4 & 6	5.37	Yes	2.57	Yes	1.56	No	0.67	No	5.58	Yes	2.58	Yes	4.88	Yes	2.89	Yes
5 & 6	4.16	Yes	1.15	No	0.61	No	0.10	No	2.31	Yes	1.07	No	3.19	Yes	1.83	No

Ten and eleven of the fifteen comparisons of mean K_M values were different when these values were derived by the v on $\frac{v}{[S]}$ and $\frac{1}{v}$ on $\frac{1}{[S]}$ plots respectively. The corresponding tests on the V_{MAX} values showed five and six differences to be significant respectively. For neither the K_M nor V_{MAX} values, derived by either plot, was there a significant difference in the results obtained during the second minute by the use of digital counter read-out or the titrigraph curve.

It appeared, therefore, that at the lowest concentration [AcCh] the value of the reaction velocity was likely to be influenced by the time at which readings were taken. The same finding applied to the K_M values. The conclusion was that the reaction velocities and the K_M values should be derived from the earliest readings which could, with convenience, be taken during the reaction. These were the readings during the second minute.

A question to be answered was whether these results were best calculated from the digital read-out of titrant added or from the titrigraph curve during this period. Except for the reaction velocity at 2.0 m mol/l [AcCh] the mean values obtained by these methods were not significantly different. The higher value obtained by digital counter when [AcCh] was 2.0 m mol/l might have reflected errors in reading the counter when there was a fast titrant delivery. Here the counter was often giving rapidly changing values at the time of reading.

The variances of results obtained during the second minute by digital read-out and titrigraph curve have been compared by variance ratio tests, the results of which are given in Table 35. These results showed that the reaction velocities at two of the four AcCh concentrations and the kinetic parameters obtained by either graphical plot had a significantly greater variance when derived from the digital read-out rather than from the slope of the titrigraph curve.

If the coefficients of variation of the reaction velocities and kinetic parameters are examined (Table 33) it will be seen that, with the exception of the K_M values, the results derived from the titrigraph curve during the second minute of reaction show coefficients of variation of 5% or less. When the digital read-out results during the second minute are compared it is seen that in all cases the corresponding coefficients are numerically greater, often greatly so. The longer the period of time over which the read-out results were calculated the more precise they became. Often this precision (Column 6, Table 33) was very high.

The overall conclusions from these experiments was that the longer the period of time over the six minutes of reaction from which results were calculated the more precise both the reaction velocities at the lower substrate concentrations and the kinetic parameters became. However, this increased precision was accompanied by a reduction in accuracy. Thus it appeared that results calculated during the second minute of the reaction should be used in future experiments and that these might be best determined from the tangent to the titrigraph curve rather than from the digital counter read-out.

Table 35

Results ('F' - values) of variance ratio tests applied to reaction velocities (v) and kinetic parameters (K_M, V_{MAX}) obtained by the use of the digital read-out or the titrigraph curve during the second minute of the AcCh-AcChE reaction. pH 7.0, 37°C.

In all tests 5, 5 df. F(theoretical); 5,5 df; P = 0.95 = 5.05
D.S. - Difference Significant.

[AcCh] m mol/l	Plot		'F' calculated from results using read-out / results using titrigraph curve.	D.S.
0.25		v	14.09	Yes
0.50		v	2.33	No
1.00		v	1.35	No
2.00		v	11.84	Yes
	$v, \frac{v}{[S]}$	K_M	13.40	Yes
	$v, \frac{v}{[S]}$	V_{MAX}	10.67	Yes
	$\frac{1}{v}, \frac{1}{[S]}$	K_M	16.00	Yes
	$\frac{1}{v}, \frac{1}{[S]}$	V_{MAX}	10.91	Yes

Calculation of the kinetic parameters K_M and V_{MAX}

The kinetic parameters K_M and V_{MAX} have been calculated for the AcCh - AcChE reaction in the present research from plots derived from two linear transformationsof the equation:-

$$v \text{ (reaction velocity)} = \frac{V_{MAX}\ [S]}{K_M + [S]} \quad \text{(equation 1, p 19)}$$

The two plots were of $\frac{1}{v}$ on $\frac{1}{[S]}$ and of v on $\frac{v}{[S]}$. The choice of these plots reflected the frequent use in biochemistry of the double reciprocal plot, $\frac{1}{v}$ on $\frac{1}{[S]}$, and the claim that the v on $\frac{v}{[S]}$ plot gave more reasonable estimates of the parameters K_M and V_{MAX} than did the $\frac{1}{v}$ on $\frac{1}{[S]}$ plot. (Colquhoun 1969,1971).

It is shown, however, in a subsequent section of this thesis (page 158) that the use of neither of these plots offered significant advantages in respect of the accuracy and precision of the derived kinetic parameters.

The use of the unweighted double reciprocal plot for the estimation of K_M and V_{MAX} has, however, been criticised by Colquhoun (1971). He also pointed out that in real experimental situations the correct weighting factors for the dependent variable would not be known because of lack of a knowledge of the population variances and means for this variable. It was felt that, in the present research, weighted regression analyses might be applied to this plot, and the influence of that weighting on the derived kinetic parameters could be tested if the errors associated with the dependent variable, reaction velocity, were known.

The earliest results for the AcCh-AcChE reaction utilising a single batch of enzyme showed that as the concentration [S] rose the coefficients of variation (cv) of the reaction velocities obtained at different substrate concentrations remained approximately constant. At concentrations [AcCh] 0.25, 0.5, 1.0 and 2.0 m mol/l the coefficients of variation were 6.64, 5.86, 6.57 and 6.39% respectively and reflected

a rise in standard deviation with increase in [S] . Thus as the weighting of variables is proportional to the reciprocal of their variance (Colquhoun, 1971) it was seen that the results obtained with the lowest concentrations [S] would be given greatest weight in plots where v was the dependent variable. In the double reciprocal plot the results ($\frac{1}{v}$) obtained at the lowest values ($\frac{1}{[S]}$) and equivalent to the highest value, v, would in turn be given greatest weight.

The variance of the reciprocal of v is given by $\frac{'var'}{\mu 4}$ where 'var' is the population variance and μ the population mean for v (Colquhoun, 1971). Here this variance and the weighting factors for $\frac{1}{v}$ were estimated from the relationship $\frac{\text{variance (v)}}{\bar{v}^4}$ as follows:-

variance (v) = $cv^2(v) \times \bar{v}^2$ where $\bar{v}$ is an experimentally determined mean for v.

$$\text{variance } (\tfrac{1}{v}) = \frac{cv^2(v) \times \bar{v}^2}{\bar{v}^4} = \frac{cv^2(v)}{\bar{v}^2}$$

Thus the weighting factor for $\frac{1}{v}$ at a given value $(\frac{1}{[S]}) = \frac{v^{-2}}{cv^2\ (v)}$

As cv (v) was constant at the different concentrations [S] the weighting factors were proportional to $\bar{v}^2$. In the double reciprocal plots based on only one value of v determined at each value [S] the weighting factors for each value $(\frac{1}{v})$ were thus given by v^2.

Using these factors weighted regression analyses (Lyall 1974) were applied to the double reciprocal plots. From the weighted intercepts were calculated the values of V_{MAX} and K_M as before. These parameters were compared with the corresponding values calculated from the non-weighted regression analyses. The null hypothesis was proposed that the differences between the corresponding values of V_{MAX} were drawn from a population of differences with a mean value of zero. This hypothesis was tested by the calculation of a value for 't'. This was also done in respect of the differences in K_M values. The value of 't' calculated for the V_{MAX} differences was 0.26

(6df) and for the K_M differences 0.06 (6df). In both cases the probability that the differences were due to chance was greater than 5%.

To test whether this lack of significant difference was seen with parameters derived from a markedly different range of reaction velocities, results were examined from four experiments where the reaction was significantly inhibited by physostigmine. The inhibited velocities were approximately 30 to 40% of the uninhibited reactions at the corresponding substrate concentrations. For the inhibited reactions the parameters Kp, the effective Michaelis constant, and Vp, the maximum reaction velocity in the presence of inhibitor, were calculated by weighted and unweighted regression analysis and the differences again compared. The value of 't' (3df) for the Kp differences was 0.57 whilst for the Vp differences it was 0.05. Thus the probabilities that these differences were due to chance was again greater than 5%.

It was concluded that the application of weighted regression analyses to the double reciprocal plot afforded no significant advantage over the use of unweighted regression analyses for the calculation of the kinetic parameters.

Characterisation of enzyme system under test.

The characterisation of the AcChE from bovine erythrocytes in respect of its reaction with AcCh as substrate, and as determined by the pH - stat method, was undertaken. The tests were carried out at 37°C and, unless otherwise stated, at pH 7.0. This pH was readily achieved and maintained during the reaction. The majority of experiments were performed at pH 7.0 as it was intended to study the reaction in the presence of the substituted benzotriazinium compounds. The formation of insoluble zwitterions by these compounds was thought to be negligible at this pH (see Cull and Scott, 1973).

1. Relationship between enzyme concentration and reaction velocity.

This relationship was examined in six experiments. Each experiment involved the reaction between a single enzyme concentration and four concentrations of substrate. The order of reaction of different substrate concentrations with[E] varied in different experiments. Three enzyme concentrations were examined, each on two occasions. The design of the experiments is outlined below

	Experiment 1.					Experiment 2.				
Day	F.F.C(E) units/ml	Order of use of substrate concentrations(m mol/l)				F.F.C(E) units/ml	Order of use of substrate concentrations(m mol/l)			
1	0.05	2.00	1.00	0.50	0.25	0.025	2.00	1.00	0.50	0.25
2	0.10	0.25	0.50	1.00	2.00	0.50	0.25	1.00	0.50	2.00
3	0.025	0.25	0.50	1.00	2.00	0.10	2.00	1.00	0.50	0.25

Results.

The mean of the two determinations of reaction velocity for each combination of [E] and [S] was calculated. This mean was plotted against [E] and a regression analysis performed between these variables for each concentration [S] . The corresponding values of V_{MAX}, as derived by the plots of v on $\frac{v}{[S]}$ and $\frac{1}{v}$ on $\frac{1}{[S]}$, were also plotted against [E] and regression analyses again applied to the variables.

The relationships between [E] and reaction velocity are

illustrated in Fig 10 . The errors of the regression lines were as follows

[AcCh] m mol/l	V_{MAX} derived by plot	Error of regression line of velocity or V_{MAX} on [E] $\left(\frac{Sb}{b}\right)$ x 100%
0.25		6.72
0.50		1.68
1.00		0.17
2.00		5.33
	v on $\frac{v}{[S]}$	6.44
	$\frac{1}{v}$ on $\frac{1}{[S]}$	7.40

b was the slope of the regression line and Sb the standard error of the slope.

It was concluded that a linear relationship existed between reaction velocity and [E] for each of the four concentrations [S] studied and for the corresponding V_{MAX} values.

In subsequent experiments a concentration [E] of 0.05 units/ml and concentrations [S] in the range 0.25 to 2.00 m mol/l were utilised as the present results pointed to the validity of determinations of enzyme activity under these circumstances (see Dixon and Webb,1964).

2. <u>Relationship between enzyme activity and substrate concentration.</u>

Two determinations of reaction velocity were made at each of the AcCh concentrations, 0.25, 0.50, 1.00, 2.00, 4.00, 8.00 and 16.00 m mol/l

<u>Results.</u>

The results are illustrated in Fig 11. This figure illustrates the characteristic inhibition of AcChE by excess AcCh substrate (see Cohen and Oosterbaan, 1963). It also confirmed that 0.25 to 2.00 m mol/l constituted a suitable range of AcCh concentrations for experiments with a concentration [E] of 0.05 units/ml.

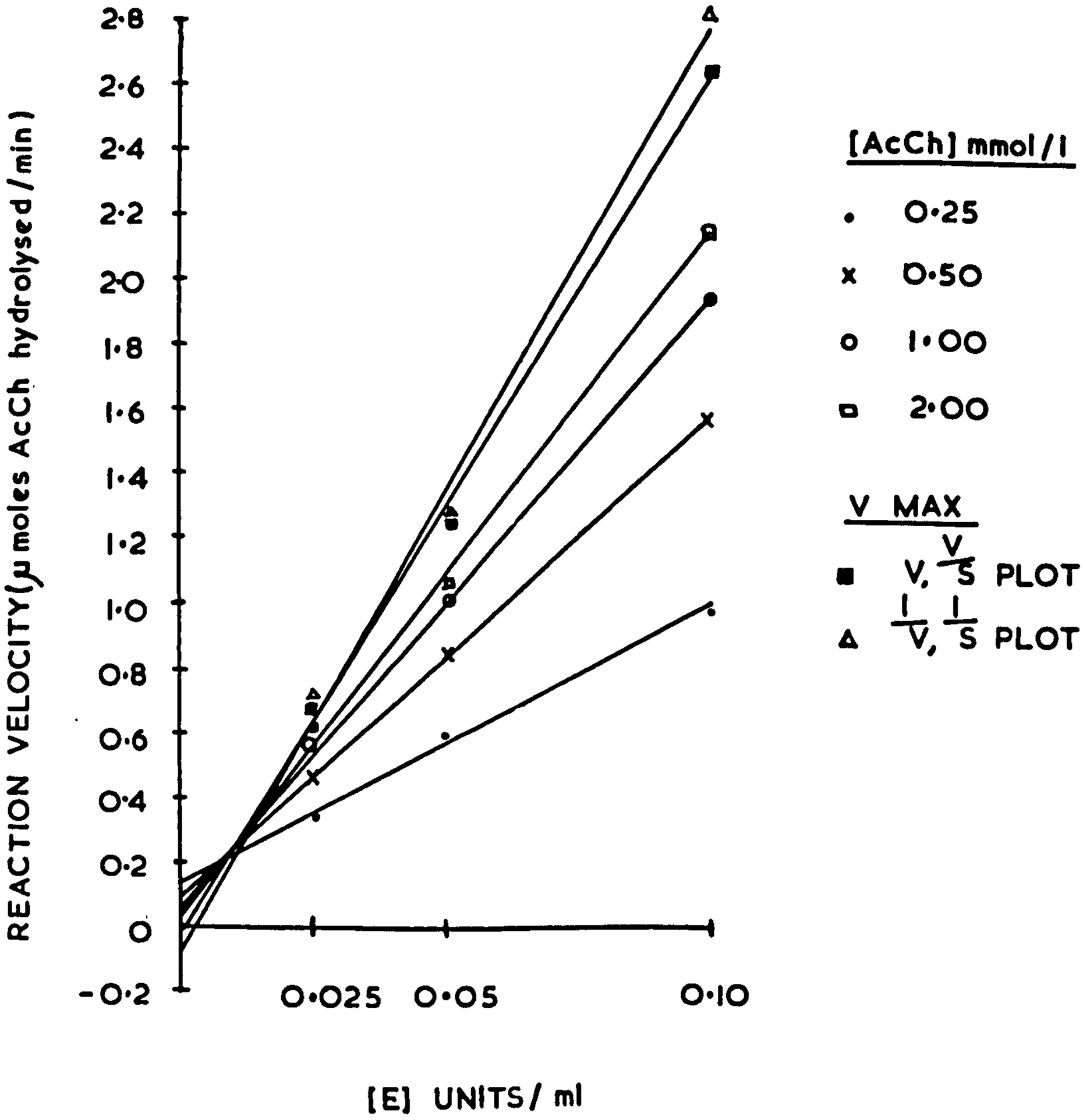


Fig 10.

Relationship between enzyme concentration [E] and reaction velocity for the AcCh-AcChE reaction.

pH - stat method. pH 7.0, 37°C.

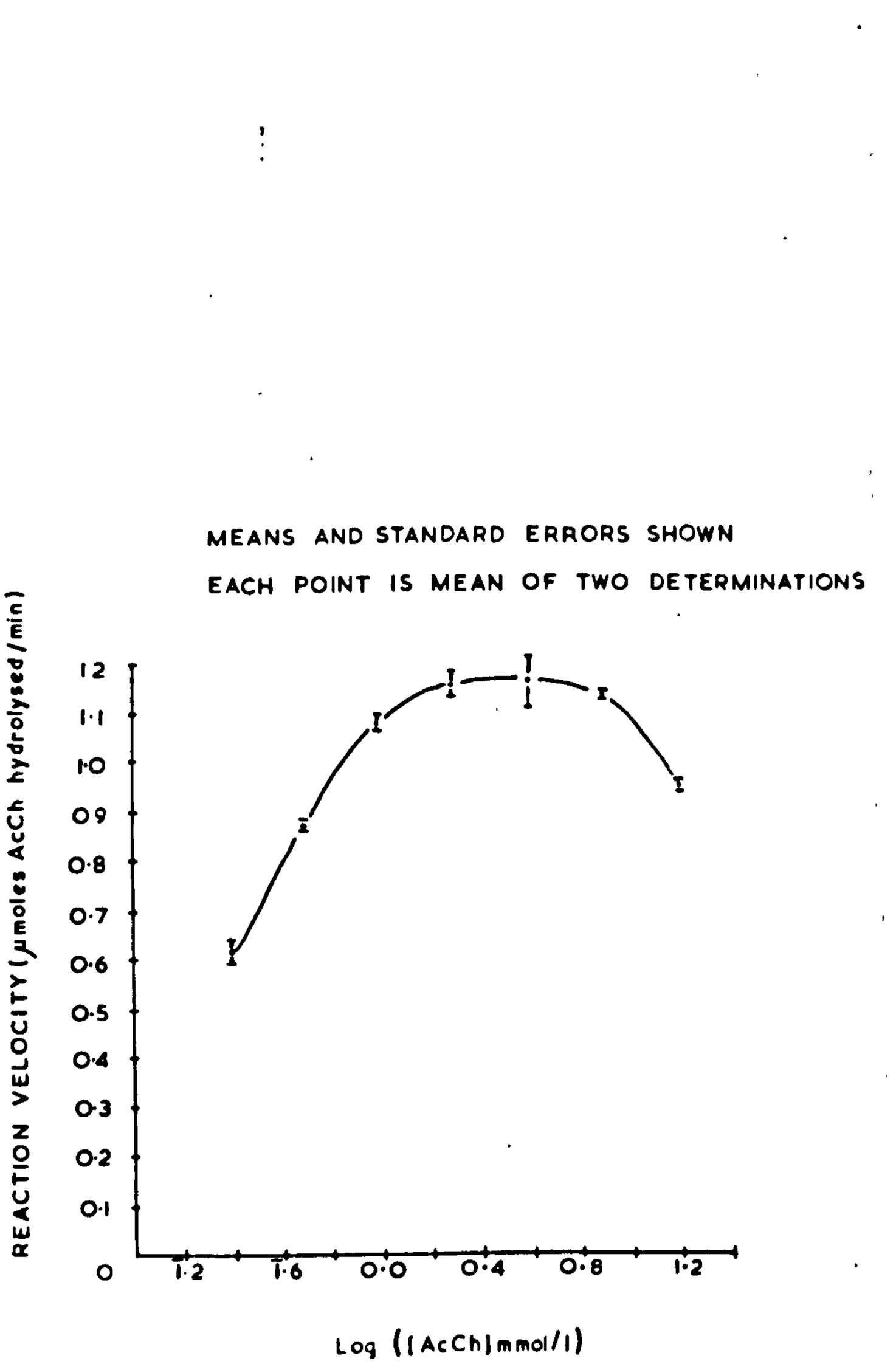


Fig 11.

Relationship between substrate concentration and reaction velocity for the AcCh-AcChE reaction.

pH - stat method pH 7.0, 37°C.

[E] - 0.05 units/ml.

3. Influence of different batches of enzyme on the results.

The pH - stat experiments performed in this research utilised several master enzyme solutions (M.E.S) prepared from different samples of commercially available enzyme. It was considered necessary to ascertain whether each M.E.S as prepared, gave values for K_M which were consistent with values reported in the literature for the reaction in order that studies with each M.E.S might be considered valid.

A comparison of reaction velocities and kinetic parameters obtained by the use of different M.E.S was also undertaken to assess any inter- M.E.S variations in activity.

Results from experiments with eight different M.E.S were collected. Different numbers of experiments were performed with the different solutions. For each batch of M.E.S the reaction velocities at each of the substrate concentrations 0.25, 0.50, 1.00 and 2.00 m mol/l were recorded. The corresponding values K_M and V_{MAX} obtained by each of the linear plots of v on $\frac{v}{[S]}$ and of $\frac{1}{v}$ on $\frac{1}{[S]}$ were also recorded.

Results.

Table36 shows these results whilst Table37 shows the results of 't' tests performed on mean K_M values obtained with different batches M.E.S. and by the plot of v on $\frac{v}{[S]}$. Table 38 shows the comparable results of tests performed on the K_M values obtained by the $\frac{1}{v}$ on $\frac{1}{[S]}$ plot whilst Tables 39 and 40show the results obtained in tests on the corresponding V_{MAX} values.

This series of Tables showed that reaction velocities may vary with the use of different batches of M.E.S but that, in general, the mean K_M values did not. None of the twenty-eight comparisons of mean K_M values derived by the v on $\frac{v}{[S]}$ plot showed significant inter-batch differences (Table 37) . Only two of the comparisons of mean K_M values derived by the $\frac{1}{v}$ on $\frac{1}{[S]}$ plot showed such differences (Table 38).

Table 36

Reaction velocities (v) and kinetic parameters (K_M, V_{MAX}) obtainsd for the AcCh-AcChE reaction by the use of different Master enzyme solutions (M.E.S) and the pH - stat method at pH 7.0 and 37°C.

[E] = 0.05 units/ml N = number of experiments performed. cv = coefficient of variation%.se = standard error
Velocities - µmoles AcCh hydrolysed/min. K_M - m mol/l

[AcCh] m mol/l			Batch M.E.S 13	14	15	16	18	19	20	21
0.25	v	N	9	2	2	5	9	8	7	7
		mean	0.5240	0.5923	0.6201	0.6591	0.6291	0.7115	0.6806	0.6350
		se	0.0116	0.0077	0.0107	0.0217	0.0111	0.0119	0.0092	0.0121
		cv	6.64	1.84	2.44	7.36	5.28	4.73	3.58	5.05
0.50	v	N	9	2	2	6	9	8	8	7
		mean	0.7222	0.8443	0.8771	0.9312	0.8376	0.9759	0.8953	0.8895
		se	0.0141	0.0246	0.0082	0.0128	0.0064	0.0079	0.0171	0.0121
		cv	5.86	4.12	1.31	3.37	2.31	2.29	5.40	3.59
1.00	v	N	9	12	15	15	9	8	8	7
		mean	0.8816	0.9746	1.0002	1.1079	1.0253	1.1885	1.1317	1.0726
		se	0.0193	0.0124	0.0130	0.0124	0.0098	0.0150	0.0186	0.0159
		cv	6.57	4.40	5.02	4.33	2.88	3.56	4.66	3.92
2.00	v	N	9	2	2	6	9	8	8	7
		mean	0.9862	1.0451	1.1151	1.2446	1.1263	1.3137	1.2126	1.2001
		se	0.0210	0.0620	0.0183	0.0101	0.0143	0.0164	0.0106	0.0272
		cv	6.39	8.39	2.31	2.00	3.81	3.53	2.48	6.00
		column	1	2	3	4	5	6	7	8

continued.....

Table 36 — continued

Plot			Batch M.E.S 13	14	15	16	18	19	20	21
$v, \frac{v}{[S]}$	K_M	N	9	2	2	5	9	8	8	7
		mean	0.2854	0.2501	0.2555	0.2938	0.2575	0.2760	0.2560	0.2865
		se	0.0143	0.0512	0.0032	0.0185	0.0108	0.0098	0.0109	0.0158
		cv	15.04	28.95	1.77	14.04	12.54	10.03	12.03	14.63
$v, \frac{v}{[S]}$	V_{MAX}	N	9	2	2	5	9	8	8	7
		mean	1.1293	1.2214	1.2833	1.4523	1.2764	1.5052	1.3818	1.3797
		se	0.0278	0.0834	0.0089	0.0168	0.0179	0.0168	0.0154	0.0258
		cv	7.38	9.65	0.98	2.59	4.21	3.16	3.16	4.96
$\frac{1}{v}, \frac{1}{[S]}$	K_M	N	9	2	2	5	9	8	8	7
		mean	0.2943	0.2705	0.2705	0.3094	0.2612	0.2820	0.2559	0.2962
		se	0.0174	0.414	0.0065	0.0256	0.0140	0.0117	0.0108	0.0125
		cv	17.79	21.62	3.40	18.48	16.07	11.71	11.98	11.19
$\frac{1}{v}, \frac{1}{[S]}$	V_{MAX}	N	9	2	2	5	9	8	8	7
		mean	1.1390	1.2488	1.3055	1.4758	1.2806	1.5143	1.3802	1.3915
		se	0.0301	0.0701	0.0044	0.0259	0.0208	0.0179	0.0157	0.0189
		cv	7.92	7.94	0.47	3.92	4.88	3.35	3.22	3.59
		column	1	2	3	4	5	6	7	8

Table 37

't' tests on mean K_M values derived by plot of v against $\frac{v}{[S]}$ from reaction between AcCh and AcChE and shown in Table 36 (Columns 1 to 8)

t test between results from M.E.S batches.	calculated 't' value	df	theoretical 't' value(P=0.95)	Difference Significant
13 and 14	0.96	9	2.26	No
13 and 15	0.94	9	2.26	No
13 and 16	0.36	12	2.18	No
13 and 18	1.56	16	2.12	No
13 and 19	0.53	15	2.13	No
13 and 20	1.61	15	2.13	No
13 and 21	0.05	14	2.14	No
14 and 15	0.11	2	4.30	No
14 and 16	1.06	5	2.57	No
14 and 18	0.24	9	2.26	No
14 and 19	0.90	8	2.31	No
14 and 20	0.19	8	2.31	No
14 and 21	0.96	7	2.36	No
15 and 16	1.24	5	2.57	No
15 and 18	0.08	9	2.26	No
15 and 19	1.00	8	2.31	No
15 and 20	0.02	8	2.31	No
15 and 21	0.99	7	2.36	No
16 and 18	1.83	12	2.18	No
16 and 19	0.94	11	2.20	No
16 and 20	1.90	11	2.20	No
16 and 21	0.30	10	2.23	No
18 and 19	1.26	15	2.13	No
18 and 20	0.10	15	2.13	No
18 and 21	1.56	14	2.14	No
19 and 20	1.37	14	2.14	No
19 and 21	0.58	13	2.16	No
20 and 21	1.62	13	2.16	No

Table 38

't' tests on mean K_M values derived by plot of $\frac{1}{v}$ against $\frac{1}{[S]}$ from reaction between AcCh and AcChE and shown in Table 36 (Columns 1 to 8)

t test between results from M.E.S batches.	calculated 't' value	df	theoretical 't' value (P=0.95)	Difference Significant
13 and 14	0.58	9	2.26	No
13 and 15	0.62	9	2.26	No
13 and 16	0.50	12	2.18	No
13 and 18	1.48	16	2.12	No
13 and 19	0.57	15	2.13	No
13 and 20	1.81	15	2.13	No
13 and 21	0.08	14	2.14	No
14 and 15	0.00	2	4.30	No
14 and 16	0.81	5	2.57	No
14 and 18	0.27	9	2.26	No
14 and 19	0.39	8	2.31	No
14 and 20	0.52	8	2.31	No
14 and 21	0.85	7	2.36	No
15 and 16	0.91	5	2.57	No
15 and 18	0.30	9	2.26	No
15 and 19	0.47	8	2.31	No
15 and 20	0.64	8	2.31	No
15 and 21	1.04	7	2.36	No
16 and 18	1.82	12	2.18	No
16 and 19	1.11	11	2.20	No
16 and 20	2.22	11	2.20	Yes
16 and 21	0.51	10	2.23	No
18 and 19	1.13	15	2.13	No
18 and 20	0.29	15	2.13	No
18 and 21	1.81	14	2.14	No
19 and 20	1.64	14	2.14	No
19 and 21	0.83	13	2.16	No
20 and 21	2.44	13	2.16	Yes

Table 39

't' test on mean V_{MAX} values derived by plot of v against $\frac{v}{[S]}$ from reaction between AcCh and AcChE and shown in Table 36 (Columns 1 to 8)

t test between results from M.E,S batches.	calculated 't' value	df	theoretical 't' value (P=0.95)	Difference Significant
13 and 14	1.34	9	2.26	No
13 and 15	2.50	9	2.26	Yes
13 and 16	8.11	12	2.18	Yes
13 and 18	4.45	16	2.12	Yes
13 and 19	11.22	15	2.13	Yes
13 and 20	7.67	15	2.13	Yes
13 and 21	6.42	14	2.14	Yes
14 and 15	0.74	2	4.30	No
14 and 16	4.41	5	2.57	Yes
14 and 18	1.10	9	2.26	No
14 and 19	5.89	8	2.31	Yes
14 and 20	3.48	8	2.31	Yes
14 and 21	2.55	7	2.36	Yes
15 and 16	5.92	5	2.57	Yes
15 and 18	0.17	9	2.26	No
15 and 19	6.28	8	2.31	Yes
15 and 20	3.04	8	2.31	Yes
15 and 21	1.89	7	2.36	No
16 and 18	6.44	12	2.18	Yes
16 and 19	2.10	11	2.20	No
16 and 20	2.98	11	2.20	Yes
16 and 21	2.15	10	2.23	No
18 and 19	9.24	15	2.13	Yes
18 and 20	4.40	15	2.13	Yes
18 and 21	3.38	14	2.14	Yes
19 and 20	5.40	14	2.14	Yes
19 and 21	4.18	13	2.16	Yes
20 and 21	0.09	13	2.16	No

Table 40

't' tests on mean V_{MAX} values derived by plot of $\frac{1}{v}$ against $\frac{1}{[S]}$ from reaction between AcCh and AcChE and shown in Table 36 (Columns 1 to 8)

t test between results from M.E.S batches.	calculated 't' value	df	theoretical 't' value (P=0.95)	Difference Significant
13 and 14	1.54	9	2.26	No
13 and 15	2.50	9	2.26	Yes
13 and 16	7.47	12	2.18	Yes
13 and 18	3.87	16	2.12	Yes
13 and 19	10.38	15	2.13	Yes
13 and 20	6.84	15	2.13	Yes
13 and 21	6.63	14	2.14	Yes
14 and 15	0.81	2	4.30	No
14 and 16	3.98	5	2.57	Yes
14 and 18	0.60	9	2.26	No
14 and 19	5.69	8	2.31	Yes
14 and 20	3.06	8	2.31	Yes
14 and 21	2.99	7	2.36	Yes
15 and 16	3.93	5	2.57	Yes
15 and 18	0.54	9	2.26	No
15 and 19	5.56	8	2.31	Yes
15 and 20	2.27	8	2.31	No
15 and 21	2.32	7	2.36	No
16 and 18	5.74	12	2.18	Yes
16 and 19	1.26	11	2.20	No
16 and 20	3.37	11	2.20	Yes
16 and 21	2.70	10	2.23	Yes
18 and 19	8.39	15	2.13	Yes
18 and 20	3.74	15	2.13	Yes
18 and 21	3.83	14	2.14	Yes
19 and 20	5.62	14	2.14	Yes
19 and 21	4.71	13	2.16	Yes
20 and 21	0.46	13	2.16	No

By contrast twenty of the twenty-eight comparisons of mean V_{MAX} values obtained by each of the graphical plots showed significant inter-batch differences (Tables 39 and 40).

These findings supported the decision that studies of the influence of any individual variable on AcChE throughout this research should be performed, where possible, on a single batch of M.E.S and that control AcCh-AcChE reactions should be an integral part of such studies.

Table 36 showed that the K_M values for all the batches M.E.S utilised were of the appropriate magnitude for the AcCh-AcChE reaction (Barlow, 1964). This Table also gave information concerning the precision of the pH - stat method and the derivation of K_M and V_{MAX} values by the two linear plots used. These matters are the subject of later discussion.

4. <u>Stability of Master enzyme solutions (M.E.S).</u>

The different batches of M.E.S were used over different periods of time and from the results which they yielded was obtained information regarding their stability. Batches 13 and 18 M.E.S were used over the longest periods, batch 18 being used over thirty-seven days. During this period nine determinations of V_{MAX} were made utilising the $\frac{1}{v}$ on $\frac{1}{[S]}$ plot. The nine V_{MAX} values (μmoles AcCh hydrolysed per minute) listed in order of determination were 1.22, 1.36, 1.35, 1.31, 1.22, 1.28, 1.32, 1.27 and 1.18. The random variation in these values and their coefficient of variation of 4.88% suggested that a M.E.S stored at $4^{o}C$ and in the dark would be stable for thirty-seven days.

Evidence that the stability of a M.E.S might not be retained for forty-five days was seen in experiments with Batch 13 M.E.S. This solution was used over thirty-one days giving a random variation in V_{MAX} values ($\frac{1}{v}$ on $\frac{1}{[S]}$plot) of 0.95 to 1.23 with a coefficient of

variation of 7.92%. Sufficient of this solution remained at the forty-fifth day for it to be tested against one concentration [S]. The chosen concentration of 1.00 m mol/l AcCh had given reaction velocities varying randomly from 0.77 to 0.95 μmoles AcCh hydrolysed per minute (coefficient of variation, 6.57%) during thirty-one days of use. On the forty-fifth day after preparation of the M.E.S the reaction velocity at this substrate concentration had fallen to a value of 0.54 μmoles AcCh hydrolysed per minute.

These findings are consistent with those of Robinson and Robinson (1968) who stated that solutions of erythrocyte AcChE stored at 0°C in the dark were stable for three to four weeks.

Precision of results

Table 36 showed that for the interaction between AcCh and AcChE, under the stated conditions, the pH - stat method allowed measurement of reaction velocities with an acceptable precision. Of the thirty-two sets of reaction velocities obtained with the use of eight batches of M.E.S, each at four concentrations [S] , twenty-six of the sets had coefficients of variation (cv) of 5% or less. The greatest cv was 8.39%.

When the sets of V_{MAX} values, as derived by the two linear plots, were examined it was found that twelve of the sixteen sets had a cv of 5% or less, the greatest cv being 9.65%.

However, when the sets of K_M values were examined it was found that only two sets had a cv less than 5%. Nine sets of values had a cv of between 10% and 15% and two sets had a cv of greater than 20%. Thus it appeared that measurements of reaction velocity could be repeated with greater precision than measurements of K_M values.

Table 41 shows the mean error of the regression lines derived by the two linear plots and from which the kinetic parameters were estimated, It is seen that for all batches of M.E.S the mean errors for the regression lines derived by the plot of v on $\frac{v}{[S]}$ were numerically the greater. When the individual corresponding pairs of regression lines were examined for each batch M.E.S it was again found that in all cases the errors were numerically greater when the v on $\frac{v}{[S]}$ plot was used. To check whether, for a given batch of M.E.S, these differences were significant the null hypothesis was proposed that they came from a population of differences with a mean value of zero. The hypothesis was tested by a calculation of 't'. The results of this calculation are given in Table 42 which shows that for six batches of M.E.S the v on $\frac{v}{[S]}$ regression line errors were significantly the greater. The lack of significant difference for the other two batches of M.E.S may merely reflect the fact that there was only one

Table 41

Comparison of errors $\left(\frac{Sb}{b}\right)$ x 100% of regression lines used for the derivation of K_M and V_{MAX} values (Table 36) and obtained by the use of two plots and different Master enzyme solutions (M.E.S).

pH stat method. pH 7.0 T = 37°C [E] = 0.05 units/ml. b = regression coefficient.
Sb = standard error of regression coefficient. v = reaction velocity [S]= substrate concentration.

Batch M.E.S	13		14		15	
Plot used	v on $\frac{v}{[S]}$	$\frac{1}{v}$ on $\frac{1}{[S]}$	v on $\frac{v}{[S]}$	$\frac{1}{v}$ on $\frac{1}{[S]}$	v on $\frac{v}{[S]}$	$\frac{1}{v}$ on $\frac{1}{[S]}$
Number of regression lines	7	7	2	2	2	2
Mean error %	15.23	9.36	19.26	10.44	11.48	6.75
Range of errors %	8.64-28.91	4.99-22.17	9.06-29.46	4.32-16.56	9.31-13.65	5.80-7.70

Batch M.E.S	16		18		19	
Plot used	v on $\frac{v}{[S]}$	$\frac{1}{v}$ on $\frac{1}{[S]}$	v om $\frac{v}{[S]}$	$\frac{1}{v}$ on $\frac{1}{[S]}$	v on $\frac{v}{[S]}$	$\frac{1}{v}$ on $\frac{1}{[S]}$
Number of regression lines	5	5	9	9	8	8
Mean error %	11.24	5.69	11.58	7.01	10.83	5.99
Range of errors %	2.32-19.23	1.34- 9.21	5.23-22.19	3.04-11.31	1.81-16.69	0.93-8.78

Batch M.E.S	20		21	
Plot used	v on $\frac{v}{[S]}$	$\frac{1}{v}$ on $\frac{1}{[S]}$	v on $\frac{v}{[S]}$	$\frac{1}{v}$ on $\frac{1}{[S]}$
Number of regression lines	8	8	7	7
Mean error %	16.13	10.39	12.74	6.50
Range of errors %	8.09-26.64	4.63-20.87	5.40-25.73	3.04-10.62

Table 42

Values of 't' calculated to test null hypothesis that the differences in errors of regression lines calculated from plots of v on $\frac{v}{[S]}$ and $\frac{1}{v}$ on $\frac{1}{[S]}$ weredrawn from a population of differences with a mean value of zero.

AcCh-AcChE reaction, pH 7.0, 37°C. pH-stat method.
v - reaction velocity [S] - substrate concentration.

Batch of Master enzyme solution.	Calculated value of 't'	df	Probability that differenceswere due to chance
13	9.81	6	<0.1%
14	4.00	1	>5.0%
15	3.89	1	>5.0%
16	2.89	4	<5.0% >2.5%
18	4.92	8	<0.5% >0.1%
19	6.33	7	<0.1%
20	8.36	7	<0.1%
21	4.99	6	<0.5% >0.1%

degree of freedom in these cases.

When, however, the kinetic parameters calculated from the regression lines and the variances amongst them were examined no significant differences were found in respect of the plots used. Table 43 shows the results of 't' tests performed both between the mean K_M values derived by the two plots and between the corresponding V_{MAX} values. Table 44 shows the results of variance ratio tests applied to the K_M and the V_{MAX} values.

The results given showed that the derivation of K_M and V_{MAX} values by the v on $\frac{v}{[S]}$ plot, under the conditions of the present experiments, offered no significant advantages over derivations by the $\frac{1}{v}$ on $\frac{1}{[S]}$ plot. In fact regression lines could be fitted to the results from the use of the latter plot with greater precision than to results from the former plot.

Table 43

Results of 't' test between mean K_M values derived by the plots v on $\frac{v}{[S]}$ and $\frac{1}{v}$ on $\frac{1}{[S]}$ and between the corresponding V_{MAX} values (from Table 36)

pH-stat method pH 7.0, 37°C [E] - 0.05 units/ml

v - reaction velocity [S] - substrate concentration.

Batch Master enzyme solution.	Calculated value of 't'	df	Theoretical value of 't' (P = 0.95)	Difference Significant
K_M values				
13	0.39	16	2.12	No
14	0.31	2	4.30	No
15	2.07	2	4.30	No
16	0.49	8	2.31	No
18	0.21	16	2.12	No
19	0.40	14	2.14	No
20	0.00	14	2.14	No
21	0.48	12	2.18	No
V_{MAX} values				
13	0.24	16	2.12	No
14	0.25	2	4.30	No
15	2.25	2	4.30	No
16	0.76	8	2.31	No
18	0.15	16	2.12	No
19	0.37	14	2.14	No
20	0.07	14	2.14	No
21	0.38	12	2.18	No

Table 44

Results of variance-ratio test ('F'-values) on K_M values determined by the plots v on $\frac{v}{[S]}$ and $\frac{1}{v}$ on $\frac{1}{[S]}$ and on the corresponding V_{MAX} values (see Table 36)

pH-stat method. pH 7.0, 37°C. [E] = 0.05 units/ml
v - reaction velocity [S] - substrate concentration.

Batch Master enzyme solution	Variance v on $\frac{v}{[S]}$ plot	df	Variance $\frac{1}{v}$ on $\frac{1}{[S]}$ plot	df	Variance ratio 'F'	Theoretical value 'F' (P=0.95)	Difference Significant
K_M values							
13	0.001841	8	0.002739	8	1.49	3.44	No
14	0.005242	1	0.003419	1	1.53	161.44	No
15	0.000020	1	0.000084	1	4.20	161.44	No
16	0.001702	4	0.003269	4	1.92	6.39	No
18	0.001043	8	0.001760	8	1.69	3.44	No
19	0.000765	7	0.001091	7	1.43	3.79	No
20	0.000947	7	0.000939	7	1.01	3.79	No
21	0.001757	6	0.001099	6	1.60	4.28	No
V_{MAX} values							
13	0.006938	8	0.008135	8	1.17	3.44	No
14	0.013890	1	0.009828	1	1.41	161.44	No
15	0.000156	1	0.000037	1	4.22	161.44	No
16	0.001416	4	0.003344	4	2.36	6.39	No
18	0.002891	8	0.003912	8	1.35	3.44	No
19	0.002262	7	0.002574	7	1.14	3.79	No
20	0.001904	7	0.001973	7	1.04	3.79	No
21	0.004673	6	0.002496	6	1.87	4.28	No

Influence of pH on the AcCh-AcChE reaction measured by the pH-stat method at 37°C.

It was considered that experiments might be performed utilising this reaction in the presence of inhibitors at pH 7.4 as well as at pH 7.0. Hence fourteen experiments were performed on seven days to compare the AcCh-AcChE reaction at pH 7.0 and pH 7.4. Experiments were performed first at pH 7.4 on alternate days. In different experiments, at a stated pH, the order of testing different substrate concentrations was altered. The design of the experiments is given in Table 45 .

Results.

The results of the experiments are shown in Table 46. This Table also shows the results of 't' tests performed on the corresponding mean reaction velocities and kinetic parameters obtained at the two pH values. It was seen that reaction velocities at each of the four substrate concentrations and the V_{MAX} values were significantly greater at pH 7.4 than at pH 7.0. The values of K_M, however, were not significantly altered by the change in pH. These results pointed to the need for separate and adequate enzyme-substrate controls in any experiments performed with inhibitors at pH 7.4.

Table 45

Design of experiments to compare the reaction between AcCh and AcChE at pH 7.0 and pH 7.4 using the pH - stat method T = 37°C

DAY	Experiment 1.					Experiment 2.				
	pH	Order of study of substrate concentrations m mol/l				pH	Order of study of substrate concentrations m mol /l			
1	7.4	2.00	1.00	0.50	0.25	7.0	2.00	1.00	0.50	0.25
2	7.0	0.50	2.00	0.25	1.00	7.4	0.50	2.00	0.25	1.00
3	7.4	0.25	0.50	1.00	2.00	7.0	0.25	0.50	1.00	2.00
4	7.0	1.00	0.25	2.00	0.50	7.4	1.00	0.25	2.00	0.50
5	7.4	2.00	1.00	0.50	0.25	7.0	2.00	1.00	0.50	0.25
6	7.0	0.50	2.00	0.25	1.00	7.4	0.50	2.00	0.25	1.00
7	7.4	0.25	0.50	1.00	2.00	7.0	0.25	0.50	1.00	2.00

Table 46

Comparison of reaction between AcCh and AcChE (0.05 units/ml) at pH 7.0 and pH 7.4 T = 37°C. pH - stat method.

	[AcCh] m mol/l								K_M (m mol/l)				V_{MAX} (μmoles AcCh hydrolysed/min)-			
	0.25		0.50		1.00		2.00		$v, \frac{v}{[S]}$ plot		$\frac{1}{v}, \frac{1}{[S]}$ plot		$v, \frac{v}{[S]}$ plot		$\frac{1}{v}\ \frac{1}{[S]}$ plot	
pH	7.0	7.4	7.0	7.4	7.0	7.4	7.0	7.4	7.0	7.4	7.0	7.4	7.0	7.4	7.0	7.4
No. of detns.	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
Mean v or K_M	0.5222	0.5716	0.7107	0.7813	0.8692	0.9776	0.9797	1.0723	0.2826	0.2939	0.2873	0.2963	1.1150	1.2433	1.1192	1.2465
se	0.0113	0.0120	0.0155	0.0195	0.0221	0.0170	0.0263	0.0178	0.0172	0.0168	0.0184	0.0146	0.0342	0.0236	0.0350	0.0244
Column	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
t test between	1 and 2		3 and 4		5 and 6		7 and 8		9 and 10		11 and 12		13 and 14		15 and 16	
t 'calc'	3.00		2.83		3.88		2.92		0.47		0.38		3.09		2.99	
D.S (P=0.95)	Yes		Yes		Yes		Yes		No		No		Yes		Yes	

t (theoretical), P = 0.95, 12 df = 2.18

v - reaction velocity - μ moles AcCh hydrolysed per min.

se - standard error of mean.

D.S - difference significant?.

An investigation of some potential sources of error in the pH-stat method as applied to the reaction between AcCh and AcChE

The vast majority of reactions between AcCh and AcChE utilising the pH-stat method, have taken place at 37°C and pH 7.0 and involved enzyme from bovine erythrocytes. It was decided, therefore, to examine possible errors arising from the use of the method in these conditions. The four following types of possible error have been assessed.

1. Possible errors arising from spontaneous hydrolysis of the substrate

Forty tests for the spontaneous hydrolysis of AcCh were performed, each over a six minute period of time. Twenty of the tests were performed at a concentration [AcCh] of 2.0 m mol/l. Fourteen of the tests were at 0.25 m mol/l, three at 0.5 m mol/l and three at 1.0 m mol/l, [AcCh]. In no case was there any detectable spontaneous hydrolysis.

An additional five tests were performed at pH 7.4, four of them at 2.0 m mol/l [AcCh] and one of them at 0.25 m mol/l [AcCh]. Again no spontaneous hydrolysis was detected.

In connection with the effect of pH on the inhibition of AcChE by benzotriazinium compounds two tests for the spontaneous hydrolysis of [AcCh], 1.0 m mol/l, were performed at both pH 7.8 and pH 8.0. Only in the latter case was any spontaneous hydrolysis detected. The hydrolysis detected was 0.05 μmoles AcCh hydrolysed per minute, approximately 4% of the corresponding enzyme catalysed reactions.

It was therefore conculuded that, for almost all the experiments performed in the present research, spontaneous hydrolysis of AcCh substrate did not constitute a measurable error.

2. Possible errors due to non-specific acid liberation by the enzyme preparation.

Checks for non-specific acid liberation due to the AcChE preparation were carried out over a four minute period of time on three hundred and twenty-seven occasions. On none of these occasions was

acid liberation detected.

Comparable checks were also carried out at pH 7.4 on fifty-eight occasions. No acid liberation was detected at this pH.

The results of the present research were thus not influenced by acid liberation due to the enzyme preparation.

3. Volume errors arising from addition of titrant to achieve the required pH end-point prior to the start of the reaction.

The volume of 0.02 N NaOH titrant added to raise the pH of the reaction mixture to the desired end-point immediately prior to the start of the reaction was recorded on one hundred and sixty-eight occasions. The mean volume added in these instances was 0.0534 ml. with a standard error of 0.0009 ml. If the mean and standard error are expressed as a percentage of 25.0 ml, the nominal volume of the reaction mixture, then the values are 0.2136 $\pm$ 0.0036%.

It was considered that this error was sufficiently small to be ignored.

4. Volume errors arising from the addition of titrant during the six minutes of reaction.

The volume of titrant added during the course of eighty-eight reactions between AcCh and AcChE, in the absence of inhibitors, was recorded. These volumes were recorded for each of four concentrations [AcCh] . The mean volumes and their standard errors are given in Table 47. This table also give the means and their standard errors as a percentage of the 25.0 ml nominal volume of reaction mixture.

As expected the volume of titrant added during the reaction increased as the substrate concentration increased. At the highest concentration [S] normally used, however, this added volume only comprised 1.45% of the nominal reaction mixture volume after six minutes of reaction. It was not considered that this error was of sufficient magnitude to require correction of the reaction velocities particularly as these were calculated from the volume of titrant added

during the second minute of the reaction.

Table 47

Volumes of 0.02N NaOH titrant added during the six minutes of AcCh-AcChE reactions at pH 7.0 and 37°C. [E] -0.05 units/ml

[AcCh] m mol/l	0.25	0.50	1.00	2.00
Number of volumes recorded.	21	21	23	23
Mean volume (ml)	0.1750	0.2604	0.3266	0.3636
Standard error of mean.	0.0033	0.0041	0.0046	0.0056
Mean volume as % of 25 ml.	0.70	1.04	1.31	1.45
Standard error as % of 25 ml.	0.0132	0.0164	0.0184	0.0224

The application of the pH -stat method to the study of the AcCh-AcChE reaction in the presence of inhibitors.

A preliminary examination of some benzotriazinium compounds for anti-acetylcholinesterase activity.

Members of the 4-phenethylamino- and 4-benzylamino- series of 2-alkyl -1,2,3- benzotriazinium compounds were tested for their ability to inhibit the reaction between AcCh (1.0 m mol/l) and AcChE (0.05 units/ ml) at pH 7.0 and 37°C. The compounds tested are listed in Table 1 and are referred to by the abbreviations given in that Table. Each compound was made up to the required concentration in reaction medium. The AcChE solutions used in the tests had been characterised in respect of their reactions with AcCh as previously described.

Each compound was tested at the three concentrations 25, 50 and 100 μmol/l. The substrate concentration was chosen because it produced a reaction rate approaching maximal but without the risk of producing enzyme inhibition (Fig 11). The contact, or incubation, time between enzyme and inhibitor prior to the addition of substrate was three minutes. This time was chosen to give all the compounds adequate time to exhibit inhibition although results from the Warburg experiments showed that PMBI and BnPI did not require incubation in order to show inhibition.

For each benzotriazinium compound duplicate determinations of reaction velocity (v') were made with the substrate in combination with each concentration $[I]$. Duplicate determinations of reaction velocity (v) were made for the substrate alone. The means of the duplicates were used to calculate the ratio $\frac{v}{v'}$ for each concentration $[I]$. A regression analysis $\frac{v}{v'}$ on $[I]$ was performed and the value of I when $\frac{v}{v'} = 2$ was found. This concentration was the I_{50} value for the compound. Similar tests were performed on physostigmine sulphate (B.D.H) which was used at concentrations 0.05, 0.10 and 0.50 μmol/l.

Members of the 4-p-tolylamino- and 4-anilino-series of benzotriazinium compounds were also screened for anti-AcChE activity with reference to physostigmine. This was done under the author's supervision by Anderson (1973). Here, however, the reaction velocities were calculated from the volume of titrant added between the second and sixth minutes of the reactions. Each compound was tested at three concentrations [I] within the range 12.5 to 100 μmol/l.

Results and discussion.

The results obtained with the 4-phenethylamino- and 4-benzylamino-series of benzotriazinium compounds and with physostigmine are shown in Table 48 . As the regression lines from which the I_{50} values were calculated were based on only three points it was not considered worth while to place confidence limits on the I_{50} values. The validity of the linear relationships used to calculate the I_{50} values was tested by determining the errors of the regression lines. These errors are also given in Table 48 .

Table 49 shows the results for the 4-p-tolylamino- and 4-anilino-series of benzotriazinium compounds obtained by Anderson (1973).

The findings in previous experiments with PMBI, AnPBI and BnPBI (Pages 7& 104) that the benzotriazinium compounds under test were relatively weak inhibitors of AcChE by comparison with physostigmine were confirmed. The variation in the activities of the compounds was approximately one and a Half times in the benzylamino-series, two and a half times in the phenethylamino-series, three times in the anilino-series and four times in the p-tolyamino series. In none of the series was a clear relationship obvious between activity and the size of the 2-alkyl substituent.

Of the four series of compounds the anilino- and benzylamino-series appeared, overall, to be the weaker though any such differences were not statistically validated.

Table 48

Results of a preliminary examination of 4-phenethylamino- and 4-benzylamino-series of 2-alkyl-1,2,3 - benzotriazinium compounds for anti-acetylcholinesterase activity.

[E] = 0.05 units/ml [S] - AcCh - 1.0 m mol/l

T = 37°C pH 7.0

Abbreviations for compounds previously given in Table 1

Compound	I_{50} (μmol/l)	p I_{50}	Error $\left(\frac{Sb}{b}\right)$ x 100% of regression line used to derive I_{50} value.
Phenethylamino series			
PMBI	78.8 *	4.10	10.76
	67.9	4.17	11.86
PEBI	48.8	4.31	7.56
PnPBI	91.0	4.04	10.00
PiPBI	35.5	4.45	9.10
PBBI	98.8	4.01	2.56
P5BI	63.5	4.20	9.09
Physostigmine sulphate	0.0989	7.00	3.13
Benzylamino series			
BMBI	139.1	3.86	5.93
BEBI	143.2	3.84	5.60
BnPBI	154.7	3.81	11.91
BiPBI	93.3	4.03	6.79
BBBI	99.9	4.00	5.60
B5BI	115.7	3.94	4.07
Physostigmine sulphate	0.0956	7.02	5.32

* A second batch of compound was used here.

p I_{50} - $\log_{10}$ reciprocal of I_{50} expressed as mol/l

Table 49

Results of a preliminary examination of 4-p-tolylamino and 4- anilino series of 2-alkyl- 1,2,3- benzotriazinium compounds for anti-acetylcholinesterase activity. (from Anderson 1973)

[E] = 0.05 units/ml [S]- AcCh - 1.0 m mol/l

T = 37°C pH 7.0

Abbreviations for compounds previously given in Table 1

Compound	I_{50}- (μ mol/l)	p I_{50}-
p-tolylamino-series		
TMBI	118	3.93
TEBI	130	3.89
TnPBI	52	4.28
TiPBI	34	4.47
TBBI	34	4.47
T5BI *	115.7	3.94
anilino series		
AMBI	122	3.91
AEBI	141	3.85
AnPBI	117	3.95
AiPBI	207	3.68
ABBI	108.7	3.96
A5BI *	75	4.12
Physostigmine sulphate	0.084	7.08

* This compound was tested by the present author.

p I_{50} - $\log_{10}$ reciprocal of I_{50} expressed as mol/l

The results gave no indication of the type of inhibition produced by the compounds nor of the magnitude of their inhibitor constants (K_i). In view of the lack of this information and the relative weakness of the inhibition it was decided to concentrate further experiments on one of the most active of the benzotriazinium compounds tested (TBBI) in order to discover something of its mode of action.

Tests to discover whether inhibition was reversible and, if so, whether it was competitive, non-competitive or mixed dictated the nature of these experiments. The mechanism of inhibition of AcChE by TBBI was to be compared with that followedby physostigmine.

Experiments to ascertain the reversibility of the anti-acetylcholinesterase activity of 2 -n-butyl - 4-p-tolylamino - 1,2,3-benzotriazinium iodide (TBBI) at 37°C and pH 7.0

The activities of four concentrations of AcCh (0.25,0.50, 1.00 and 2.00 m mol/l) were determined in the presence of AcChE (0.05 units/ml) at 37°C and pH 7.0. From these results it was ascertained that a K_M value of the appropriate order for the AcCh-AcChE reaction was obtained. These tests were repeated with fresh substrate solutions and in the presence of TBBI (100 μmol/l). The percentage inhibition of enzyme was then calculated for each concentration [AcCh] . Each of the four solutions of inhibited enzyme was dialysed against reaction medium at 4°C in the dark. The dialysis involved the placing of a solution in cellulose tubing (Visking - 32/32") and the immersion of the tubing in 500 ml reaction medium. Five changes each of 500 ml of reaction medium were effected over twenty-four hours. After twenty-four hours fresh control reaction velocities were determined at 37°C for the four concentration [AcCh] . The activities of the dialysed solutions were also determined at 37°C after addition of AcCh to give the same concentration [AcCh] in the solution as that tested before dialysis. By reference to the fresh control reaction velocities the percentage of inhibited enzyme activity recovered by dialysis was calculated. The results are given in Table 50 (a).

This experiment was repeated but with a dialysis involving five manual changes of reaction medium each of 1500 ml in the twenty-four hours; the results are given in Table 50 (b).

Two further experiments were performed but in which only one substrate concentration (2.0 m mol/l) was employed. In the first of these experiments the reaction medium used as the dialysis solution was changed by continuous flow. The continuous flow was of 6 ml reaction medium per minute and was achieved by passing the

reaction medium through a peristaltic pump. The result is given in Table 50 (c). The second of the experiments involved twelve manual changes of reaction medium, each of 1500 ml, in forty-eight hours. The result is shown in Table 50(d).

Table50 showed that by the dialysis techniques described the inhibition of AcChE produced by TBBI was reversible. The extent of this reversal varied with the technique adopted. Table50 also showed the good reproducibility of the inhibition produced over the four tests at the 2.0 m mol/l substrate concentration.

Table 50

Results of experiments to illustrate the reversible inhibition of AcChE by TBBI (100 μ mol/l) at 37°C and pH 7.0.

Enzyme-inhibitor complex dialysed against reaction medium at 4°C.

(a) Experiments where dialysis involved five changes, each of 500 ml, of reaction medium in twenty-four hours.

F.F.C AcCh m mol/l	2.0	1.0	0.5	0.25
% inhibition of enzyme before dialysis	61.90	65.57	70.77	77.01
% inhibition of enzyme after dialysis	33.13	37.97	33.91	30.29
% lost activity recovered after dialysis	46.48	42.09	52.08	60.67

(b) Experiments where dialysis involved five changes or three changes*, each of 1500 ml, of reaction medium in twenty-four hours.

F.F.C AcCh m mol/l	2.0	1.0	0.5*	0.25
% inhibition of enzyme before dialysis	63.27	60.28	70.00	76.14
% inhibition of enzyme after dialysis	28.09	14.99	54.11	33.40
% lost activity recovered after dialysis	55.60	75.13	22.70*	56.13

(c) Experiment in which dialysis was by continuous flow of reaction medium (6ml/min) for forty-eight hours.

F.F.C AcCh m mol/l	2.0
% inhibition of enzyme before dialysis	61.96
% inhibition of enzyme after dialysis	20.83
% lost activity recovered after dialysis	66.38

(d) Experiment where dialysis involved twelve changes, each of 1500 ml, of reaction medium in forty-eight hours.

F.F.C AcCh m mol/l	2.0
% inhibition of enzyme before dialysis	60.13
% inhibition of enzyme after dialysis	8.20
% lost activity recovered after dialysis	86.36

Experiments to illustrate the kinetics and mechanism of the inhibition of AcChE by physostigmine.

It has been shown that the kinetics and mechanism of the inhibition of AcChE produced by physostigmine may alter. Lengthening of the time of incubation between enzyme and physostigmine prior to substrate addition can alter the inhibition from competitive to non-competitive (Robinson and Robinson,1968; Stein and Lewis,1969). It was considered desirable, therefore, to perform a limited number of experiments to ascertain whether the method adopted in the present research could adequately illustrate the kinetics and mechanism of physostigmine-induced inhibition prior to any investigation of the mechanism of AcChE inhibition induced by benzotriazinium compounds.

Twelve experiments were performed to investigate the interactions between AcCh, AcChE and physostigmine at pH 7.0 and 37°C using an enzyme concentration of 0.05 units/ml. The physostigmine concentration used in these experiments was 0.1 μmol/l as it had been previously shown (Tables48&49) that this concentration gave a significant degree of enzyme inhibition (approximately 50%) under the chosen experimental conditions.

The twelve experiments were divided into three sets of four experiments. In one set the AcChE - physostigmine incubation time was six minutes whilst in the second set the incubation time was three minutes. In the third set the incubation time was minus thirty seconds, that is the physostigmine was added to the reaction mixture thirty seconds after the start of the AcCh-AcChE reaction.

Each experiment involved the determination of the velocities of reaction between four concentrations of AcCh (0.25, 0.50, 1.00 and 2.00 m mol/l) in the presence (v^1) and the absence (v) of physostigmine. In each set of four experiments the order of reaction of the substrate concentrations was varied according to a Latin Square design both when the inhibitor was absent and when it was present.

Results and Discussion.

From the reaction velocities determined in each experiment the kinetic parameters K_M, V_{MAX}, Kp and Vp were determined (see Table 51). Kp and Vp represented the 'effective' Michaelis constant and the maximum reaction velocity respectively for the AcCh-AcChE reaction in the presence of the inhibitor (Dixon and Webb,1964). The four parameters were derived from each experiment by the application of regression analyses to the plots of v on $\frac{v}{S}$ and v^1 on $\frac{v^1}{S}$. They were also derived by the plots of $\frac{1}{v}$ on $[\frac{1}{S}]$ and $\frac{1}{v^1}$ on $[\frac{1}{S}]$. The forms of these plots in the presence of inhibitors effecting competitive, non-competitive and mixed inhibition are well documented (see Dixon and Webb,1964; Zeffren and Hall 1973).

As competitive enzyme inhibitors affect the affinity of the enzyme for the substrate the values of the parameters K_M and Kp should be different. Conversely non-competitive inhibitors, which do not affect the enzyme-substrate combination, cause a reduction in V_{MAX}, which should thus differ from Vp. A compound producing mixed inhibition will alter both K_M and V_{MAX}.

When the results from individual experiments were examined some trends were noted. For experiments where the inhibitor incubation time was minus thirty seconds it was found that the value of Kp was always larger than the corresponding value K_M whichever plot was used in their derivation. This was also true of experiments where the incubation time was three minutes. In experiments where the incubation time was either three minutes or six minutes the value of V_{MAX} was always bigger that the corresponding value Vp.

For each set of results, therefore, values of 't' were calculated from the differences between corresponding values Kp and K_M and between corresponding values V_{MAX} and Vp. The values of 't' were used to test the null hypothesis that the differences were from populations of differences with universal means of zero and were thus due to

Table 51

Kinetic parameters derived for the AcCh-AcChE reaction in the presence and absence of physostigmine(I) and with different incubation times between AcChE and (I).

Kp - Effective Michaelis constant when [I] = 0.1 μmol/l Vp - Maximum reaction velocity when [I] =0.1 μmol/l [E] = 0.05 units/ml. T = 37°C. pH 7.0 Each value is the mean of 4 determinations. K_M and Kp expressed as m mol/l. V_{MAX} and Vp as μmoles AcCh hydrolysed per minute.

E - I incubation time(min).		Parameters derived by plot							
		v on $\frac{v}{[S]}$				$\frac{1}{v}$ on $\frac{1}{[S]}$			
		K_M	Kp	V_{MAX}	Vp	K_M	Kp	V_{MAX}	Vp
-0.5	Mean	0.2996	0.4009	1.4010	1.4840	0.3016	0.4504	1.4028	1.5507
	Standard error	0.0221	0.0310	0.0442	0.0486	0.0190	0.0433	0.0337	0.0488
+3.0	Mean	0.2540	0.4217	1.3765	0.8010	0.2555	0.4822	1.3770	0.8466
	Standard error	0.0134	0.0394	0.0156	0.0247	0.0106	0.0272	0.0148	0.0250
+6.0	Mean	0.2846	0.2597	1.4053	0.5146	0.2931	0.2732	1.4142	0.5210
	Standard error	0.0291	0.0254	0.0419	0.0187	0.0215	0.0313	0.0282	0.0156
	Column	1	2	3	4	5	6	7	8

chance. The results of these tests are given in Table 52 .

It was seen that the results when the incubation time was six minutes were clear. They showed a significant difference between V_{MAX} and Vp derived by either plot but not between K_M and Kp and were consistent with non-competitive inhibition. When the inhibitor incubation time was three minutes both Kp and K_M values and V_{MAX} and Vp values were significantly different. These findings were consistent with the production of mixed inhibition.

When the inhibitor incubation time was minus thirty seconds no significant differences were found between Kp and K_M values despite the fact that the former were always numerically larger. A significant difference in these values might have been expected indicating competitive antagonism (Robinson and Robinson 1968; Stein and Lewis, 1969). When 't' tests were performed between the mean of these K_M and the mean of these Kp values a significant difference was in fact found using parameters calculated both by the v on $\frac{v}{[S]}$ plot ('t', calculated = 2.66) and the double reciprocal plot ('t', calculated = 3.15). These 't' values were compared with a theoretical value of 2.45 for six degrees of freedom and a 95% probability level.

The differences in the results of the two different applications of the 't' test might have reflected the fewer degrees of freedom involved in the comparison of differences. Additionally the chosen physostigmine concentration might not have been high enough to produce a significant degree of competitive inhibition when added after the start of the AcCh-AcChE reaction.

Despite the equivocal nature of the results after minus thirty seconds incubation it was clear that the kinetics and mechanism of the inhibition of AcChE produced by physostigmine was changing with the change in incubation time. A graphical representation of this change is given in Fig.12.

Table 52

Calculated values of 't' for differences between K_M and Kp values and between V_{MAX} and Vp values derived from the AcCh-AcChE reaction in the presence and absence of physostigmine at pH 7.0 and 37°C.

test between	Inhibitor incubation time(min)	plot used to derive parameters	calculated value 't'	df	Probability that differences due to chance
Kp and K_M	-0.5	v on $\frac{v}{[S]}$	2.40	3	>5%
V_{MAX} and Vp	-0.5	v on $\frac{v}{[S]}$	2.73	3	>5%
Kp and K_M	-0.5	$\frac{1}{v}$ on $\frac{1}{[S]}$	2.44	3	>5%
V_{MAX} and Vp	-0.5	$\frac{1}{v}$ on $\frac{1}{[S]}$	2.71	3	>5%
Kp and K_M	+3.0	v on $\frac{v}{[S]}$	5.04	3	<2% >1%
V_{MAX} and Vp	+3.0	v on $\frac{v}{[S]}$	41.91	3	<0.1%
Kp and K_M	+3.0	$\frac{1}{v}$ on $\frac{1}{[S]}$	10.85	3	<0.5% >0.1%
V_{MAX} and Vp	+3.0	$\frac{1}{v}$ on $\frac{1}{[S]}$	41.97	3	<0.1%
Kp and K_M	+6.0	v on $\frac{v}{[S]}$	0.52	3	>5%
V_{MAX} and Vp ,	+6.0	v on $\frac{v}{[S]}$	17.10	3	<0.1%
Kp and K_M	+6.0	$\frac{1}{v}$ on $\frac{1}{[S]}$	0.46	3	> 5%
V_{MAX} and Vp	+6.0	$\frac{1}{v}$ on $\frac{1}{[S]}$	25.22	3	<0.1%

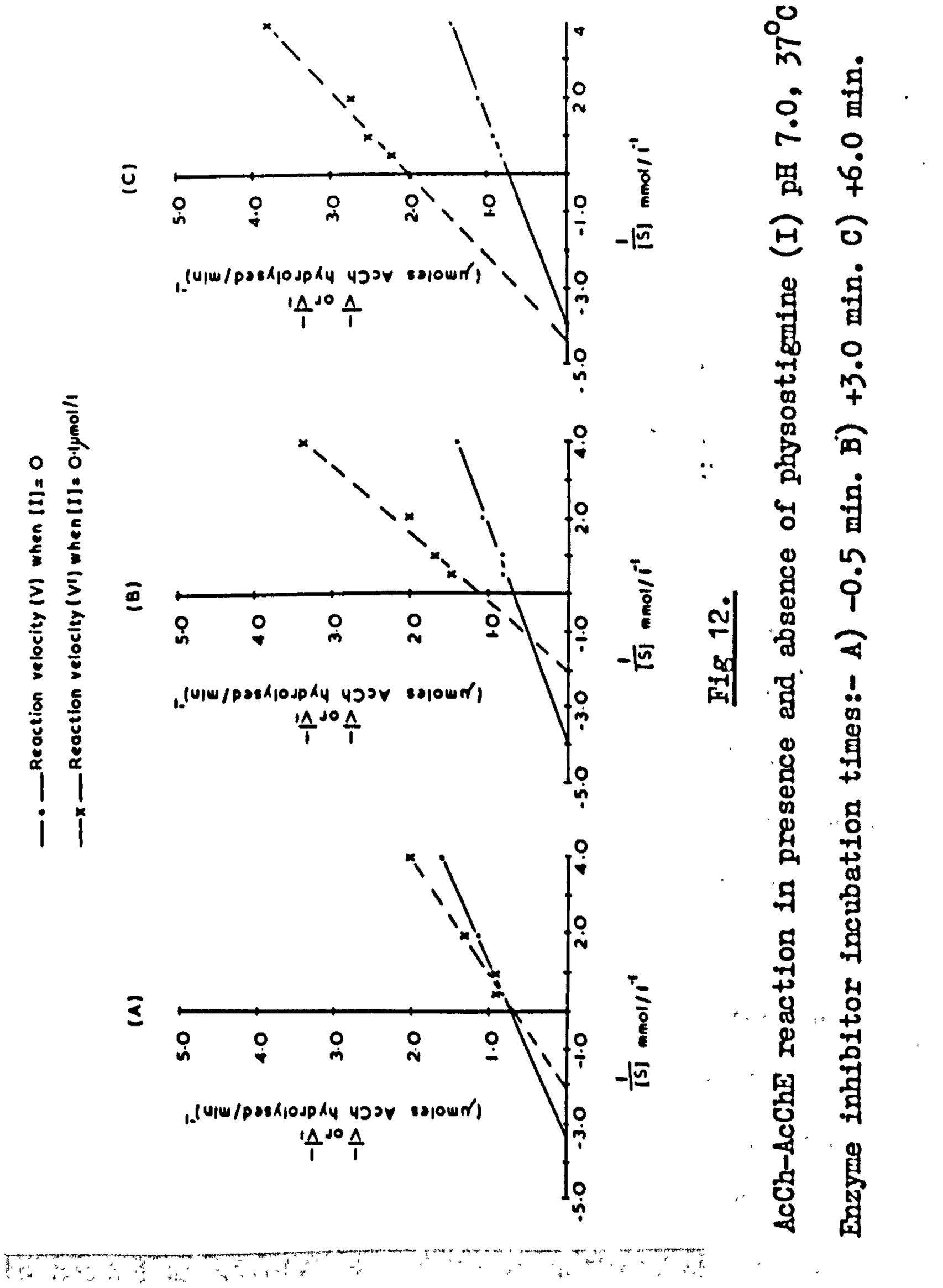


Fig 12.

AcCh–AcChE reaction in presence and absence of physostigmine (I) pH 7.0, 37°C

Enzyme inhibitor incubation times:- A) −0.5 min. B) +3.0 min. C) +6.0 min.

Values for the inhibition constant, K_i, were calculated from the double reciprocal plots relating reaction velocity and substrate concentration. The calculations involved measurement of both the slope and the intercept of the $\frac{1}{v}_1$ on $\frac{1}{[S]}$ plot. (see Zeffren and Hall, 1973).

For both fully competitive and fully non-competitive inhibitors the slope of the plot is related to K_i by the expression:-

$$\text{Slope} = \frac{K_M \left(1 + \frac{[I]}{K_i}\right)}{V_{MAX}}$$

For the fully competitive inhibitor the intercept on the abscissa, $-\frac{1}{Kp}$, is related to K_i by the expression:-

$$K_i = \frac{[I]}{\frac{Kp}{K_M} - 1}$$

In the case of fully non-competitive inhibitors the intercept on the ordinate $\frac{1}{V_p}$, is related to K_i by the expression:-

$$K_i = \frac{[I]}{\frac{V_{MAX}}{V_p} - 1}$$

The mean K_i values calculated from results obtained after both the minus thirty seconds incubation time and the six minute incubation time are given in Table 53 . This Table suggested that the strength of inhibition, as represented by a numerical decline in K_i value, increased with the increase in incubation time and so confirmed the findings of Robinson and Robinson (1968) and Stein and Lewis (1969).

However, when the mean K_i values calculated from the intercepts of the plots after the two periods of incubation were compared by 't' test the difference was not found to be significant ('t',calculated = 1.54, 6df). Similarly a comparison of mean K_i values derived from the slopes of the plots showed no significant difference ('t', calculated = 1.82,6df). The lack of significant difference in these cases reflected the large errors associated with the results obtained after minus thirty seconds incubation. It was noted that the difference in K_i values shown by Robinson and Robinson, (1968) after different periods

Table 53

Mean inhibition constants (K_i) determined for the physostigmine (I) - AcChE (E) reaction at pH 7.0 and 37°C

Each value is the mean of four determinations.

v' - reaction velocity for the AcCh-AcChE reaction in the presence of I .

[S] - [AcCh] m mol/l

Time of incubation between E and I (mins)	Type of inhibition	Part of graph of $\frac{1}{v'}$ on $\frac{1}{[S]}$ used in calculation	Mean K_i value (μmol/l)	Standard error of mean
-0.50	Competitive	Abscissa intercept	0.500	0.287
-0.50	Competitive	Slope	0.670	0.329
+6.0	Non-competitive	Ordinate intercept	0.059	0.003
+6.0	Non-competitive	Slope	0.072	0.012

of incubation were not statistically validated.

It was seen from Table 53 that, after a given period of inhibitor incubation, the mean K_i values determined from slopes of the double reciprocal plots were both numerically greater than those determined from the intercepts of the plots and also had numerically larger errors. 't' tests showed no significant differences between these mean K_i values. The calculated values of 't' were 0.39 (6df) after minus thirty seconds incubation and 1.02 (6df) after six minute incubation. These values were compared with a theoretical value of 't' of 2.45 for 6df and P = 0.95.

Variance-ratio tests were performed on the K_i values calculated after each of the incubation periods. It was found that the variance was significantly greater when derived from the slope rather than the intercepts after six minutes inhibitor incubation ('F' calculated 13.02) but that the corresponding variances of the results after minus thirty-seconds incubation were not significantly different ('F' calculated 1.31). The calculated values for 'F' were compared with a theoretical value for 3 and 3 df at P = 0.95 of 9.28.

The value obtained from the abscissa intercept for K_i after minus thirty seconds incubation (0.50 μmol/l) was in close agreement with the value of 0.55 μmol/l given by Robinson and Robinson (1968) for the result after one minute incubation with 0.1438 μmol/l physostigmine at pH 7.4 and 25°C. Dale and Robinson (1970) derived a K_i value of 0.51 μmol/l for (-)-physostigmine again after one minute incubation and at pH 7.4 and 25°C.

It was considered that the results from the present experiments were in sufficient agreement with the results from the earlier papers quoted to justify proceeding to a comparable investigation of the benzotriazinium compounds.

Experiments to illustrate the kinetics and mechanism of the inhibition of AcChE by benzotriazinium compounds.

(a) Experiments with 2-n-butyl - 4-p-tolylamino - 1,2,3 - benzotriazinium iodide.

Five experiments were performed to investigate the interactions between AcCh, AcChE and 2-n-butyl -4-p-tolylamino-1,2,3-benzotriazinium iodide (TBBI) (I) at pH 7.0, 37°C and using an enzyme concentration of 0.05 units/ml.

In each experiment the inhibited enzyme reaction rate (v^1) was compared with the uninhibited reaction rate (v) at four substrate concentrations (0.25, 0.50, 1.00 and 2.00 m mol/l). The order of reaction of these concentrations in the presence and absence of inhibitor was varied between experiments.

In two of the experiments the enzyme reaction was studied at the three concentrations [I] of 25, 50 and 100 μmol/l. The three remaining experiments utilised a single inhibitor concentration [I] of 25 μmol/l.

The enzyme inhibitor incubation time was three minutes in all experiments.

Results.

Table 54 shows the mean percentage inhibition and its error produced by TBBI at each combination of substrate and inhibitor concentration tested. Inspection of this Table suggested an element of competition between substrate and inhibitor particularly at the higher concentrations of the latter.

The results were further analysed in three different ways:-

(i) By the method of Dixon (1953)

This method was applied to the results from the two experiments in which [I] was varied. It involved plots of $\frac{1}{v_1}$ against [I] at the different substrate concentrations. The resulting lines intersect above the abscissa at a value of [I] equal to $-K_i$ in the case of

Table 54

Mean percentage inhibition of AcChE produced by TBBI at pH 7.0 and 37°C.

Figures in brackets refer to number of determinations.

[TBBI] μmol/l	[AcCh] m mol/l	2.0	1.0	0.5	0.25
25	Mean	40.97 (5)	42.44 (4)	44.81 (5)	49.31 (5)
	Standard error	0.60	2.18	1.24	2.17
50	Mean	54.80 (2)	58.30 (2)	59.49 (2)	61.54 (2)
	Standard error	1.27	1.05	1.88	0.30
100	Mean	62.59 (2)	62.93 (2)	70.39 (2)	76.58 (2)
	Standard error	0.69	2.65	0.39	0.44

competitive inhibitors. For non-competitive inhibitors the intersection point is on the abscissa at a value equal to $-K_i$ (Dixon 1953). An alternative method of deriving the value of K_i in the competitive case involves the drawing of a horizontal line on the graph at a value of $\frac{1}{V_{MAX}}$ above the abscissa. This line should intersect the lines drawn for the $\frac{1}{v}$ against $[I]$ plots above the abscissa at the value equivalent to K_i (Dixon, 1953). For this to be possible V_{MAX}, in the absence of inhibitor, had to be determined as part of the same experiment.

Regression analyses were applied to the plots of $\frac{1}{v}$ against $[I]$. The results obtained in one of the experiments are illustrated in Fig 13. From such lines the inhibition produced by TBBI appeared to be competitive. The assumption was made that the inhibition produced by TBBI was, in fact, competitive and values for K_i calculated accordingly. The mean of seven values for K_i calculated from the intercepts of the $\frac{1}{v}$ values with the regression lines was 40.4 μmol/l with a V_{MAX} standard error of 5.5 μmol/l. The K_i values were also calculated from the intercepts of the regression lines at 2.0 m mol/l substrate concentration with those at the other substrate concentrations in each experiment. Here the mean K_i value was 32.3 μ mol/l from five determinations, the standard error of the mean being 10.8 μmol/l.

(ii) By the method of Hunter and Downs (1945).

This method permits the assessment of the type of inhibition and the calculation of K_i by the combination of results from experiments with inhibitors at variable concentrations $[S]$ and $[I]$. A plot of $[I] \times \frac{v}{v - v'}$, against $[S]$ for a competitive inhibitor gives a straight line (slope $\frac{K_i}{K_M}$) cutting the ordinate at a value equivalent to K_i. For non-competitive inhibitors a straight line, parallel to the abscissa again cutting the ordinate at a value equivalent to K_i is obtained.

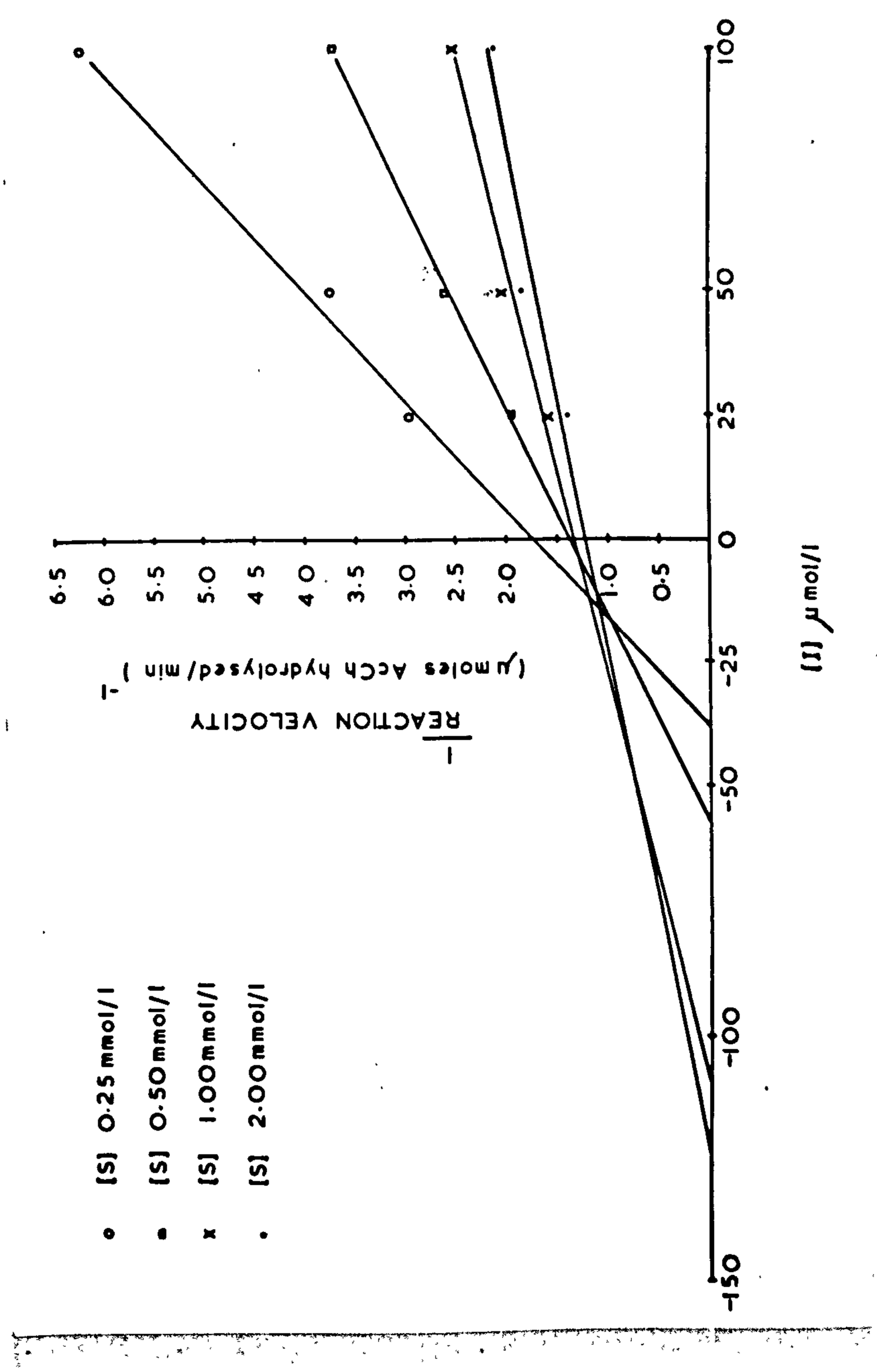


Fig 13.

Influence of TBBI (I) on the AcCh-AcChE reaction at different concentrations of AcCh(S). pH 7.0, 37°C.

The mean values calculated from the function $[I] \times \frac{v^1}{v - v^1}$ are given in Table 55 in relation to the concentrations $[I]$ and $[S]$. This Table also shows the results of 't' tests between the mean values obtained with the different concentrations of inhibitor at each concentration of substrate.

Table 55 showed that at all concentrations $[I]$ the mean value for $[I] \times \frac{v^1}{v - v^1}$ rose with increase in substrate concentration. The rise appeared to be greatest at the 100 μmol/l concentration of TBBI. This was confirmed by the 't' tests which showed that when $[S]$ was 2.0 m mol/l the function was significantly different at each concentration $[I]$. At a concentration of $[S]$ equal to either 1.0 - or 0.5 m mol/l the function when $[I]$ was 100 μmol/l was significantly different from the function when $[I]$ was 25 μmol/l. This finding suggested that the inhibition produced by 100 μmol/l TBBI was more competitive in nature than the inhibition produced at the lower TBBI concentrations.

Regression analyses were applied to plots of $[I] \times \frac{v^1}{v - v^1}$ on $[S]$ as follows:-

(a) When only results obtained with $[I]$ equal to 25μmol/l were included.

(b) When only results obtained with $[I]$ equal to 50μmol/l were included.

(c) When only results obtained with $[I]$ equal to 100μmol/l were included.

(d) When the results obtained with $[I]$ equal to 25 - and 50μ mol/l were included.

(e) When the results obtained with the three concentrations $[I]$ were combined.

The resultant regression lines are illustrated in Fig 14. The regression line (c) for results at the 100 μmol/l inhibitor

Table 55

Mean values for the function $[I] \times \frac{v'}{v-v'}$ from the AcCh-AcChE reaction in the presence (v') and the absence (v) of TBBI (I) at pH 7.0 and 37°C.

[I] μmol/l	[AcCh] m mol/l	2.0	1.0	0.5	0.25
25	mean (μmol/l)	36.07 (5)	34.40 (4)	30.96 (5)	26.13 (5)
	standard error	0.90	3.37	1.53	2.43
	result	1	4	7	10
50	mean	41.30 (2)	35.79 (2)	34.13 (2)	31.25 (2)
	standard error	2.11	1.54	2.66	0.40
	result	2	5	8	11
100	mean	59.80 (2)	59.21 (2)	42.08 (2)	30.59 (2)
	standard error	1.75	6.69	0.78	0.74
	result	3	6	9	12

Figures in brackets refer to number of determinations.

't' test between results	't' (calc).	df	't' (theo) P=0.95	't' test between results	't' (calc).	df	't' (theo) P=0.95
1 and 2*	2.79	5	2.57	7 and 8	1.09	5	2.57
1 and 3*	13.43	5	2.57	7 and 9*	4.29	5	2.57
2 and 3*	6.75	2	4.30	8 and 9	2.87	2	4.30
4 and 5	0.27	4	2.78	10 and 11	1.26	5	2.57
4 and 6*	3.81	4	2.78	10 and 12	1.09	5	2.57
5 and 6	3.41	2	4.30	11 and 12	0.78	2	4.30

* Differences significant (P = 0.95)

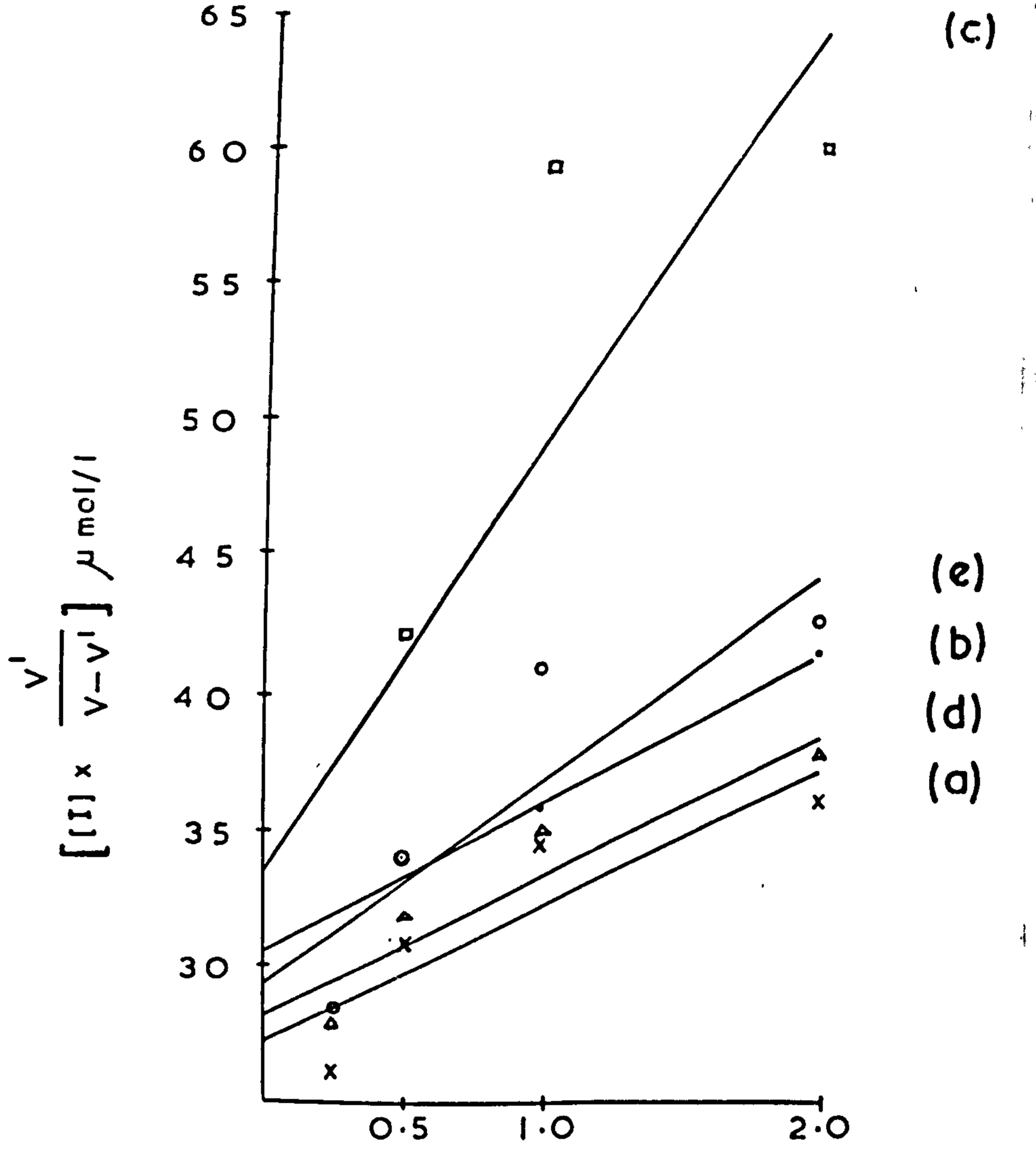


Fig 14.

Graphs showing the effect of TBBI (I) on the AcCh (S)-AcChE reaction calculated by the method of Hunter and Downs. (1945). pH 7.0, 37°C.

For explanation of labels (a) to (e) see p. 188.

concentration in particular, suggested competitive inhibition. From the intercepts of these five lines with the ordinate estimates for the K_i values were as follows:-

(a) 27.2 μmol/l

(b) 30.6 μmol/l

(c) 33.4 μmol/l

(d) 28.1 μmol/l

(e) 29.3 μmol/l

(iii) By double reciprocal plots.

In all experiments double reciprocal plots of $\frac{1}{v}$ on $\frac{1}{[S]}$ and $\frac{1}{v}$ on $\frac{1}{[S]}$ were applied to the reactions in the presence and absence of inhibitor. Regression analyses were applied to these plots and from the intercepts of the regression lines values for the kinetic parameters K_M, Kp, V_{MAX} and Vp were determined. Mean values for these parameters are shown in Table 56. The effect of increasing inhibitor concentration on the plot shown in Fig 15.

Inspection of the results obtained showed that in each experiment the value of Kp was greater than the corresponding value K_M and the value V_{MAX} was greater than the corresponding value Vp. Values of 't' were calculated from these differences and the probabilities were estimated that the differences were due to chance. The results of these tests are shown in Table 57.

It was seen that at all three concentrations [I] the values K_M and Kp were significantly different. So, too, were the corresponding values V_{MAX} and Vp. These results suggested that TBBI, under the conditions of the test in fact produced mixed inhibition of the AcChE.

Inspection of Fig 15 and the mean Kp and Vp values given in Table 56 again suggested that the action of 100 μmol/l TBBI was significantly more competitive than the action of the lower concentrations. This suggestion was tested by the application of 't' tests to the mean Kp values obtained at different concentrations [I]

Table 56

Mean kinetic parameters determined for the AcCh-AcChE reaction in the absence (K_M, V_{MAX}) and presence (Kp, Vp) of TBBI at pH 7.0 and 37°C.

[E] = 0.05 units/ml.

[TBBI] mol/l		K_M m mol/l	Kp m mol/l	V_{MAX} μmoles AcCh hydrolysed per minute	Vp μmoles AcCh hydrolysed per minute
25	N	5	5	5	5
	$\bar{x}$	0.2625	0.3851	1.3148	0.8225
	se	0.0115	0.0341	0.0364	0.0247
50	N	2	2	2	2
	$\bar{x}$	0.2736	0.3780	1.3967	0.6429
	se	0.0094	0.0161	0.0251	0.0181
100	N	2	2	2	2
	$\bar{x}$	0.2736	0.8921	1.3967	0.7225
	se	0.0094	0.0252	0.0251	0.0051

N = Number of observations

$\bar{x}$ = mean parameter

se = standard error of mean,

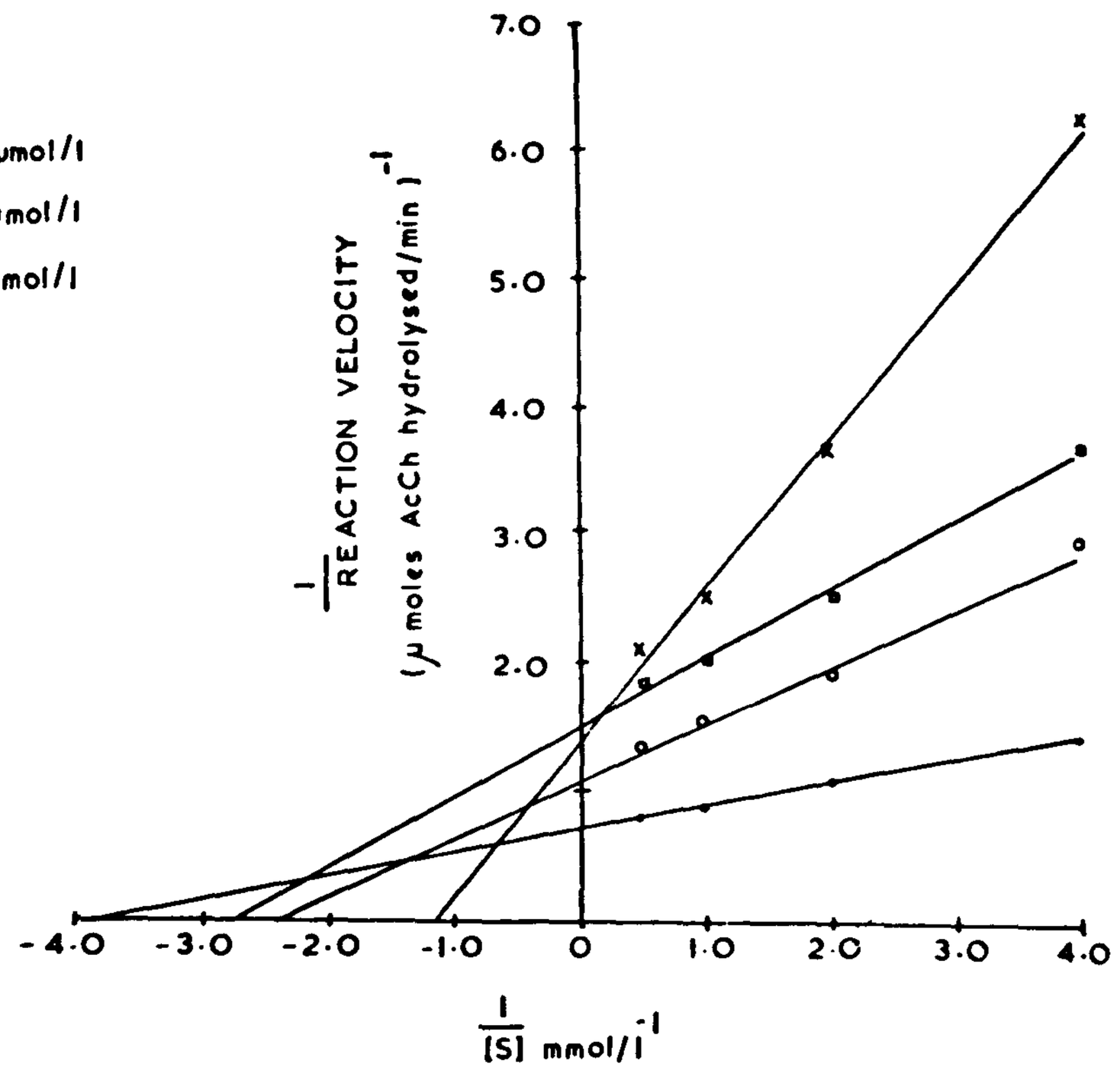


Fig 15.

Effect of increasing concentrations of TBBI (I) on AcCh(S) - AcChE reaction shown by the double reciprocal plot of $\frac{1}{v}$ on $\frac{1}{[S]}$. pH 7.0, 37°C.

Table 57

Values for 't' calculated from the differences between Kp and K_M and V_{MAX} and Vp from reactions between AcCh and AcChE at pH 7.0 and 37°C in the presence and absence of TBBI.

[TBBI] mol/l	test between differences in	calculated value of 't'	df	Probability that differences are due to chance.
2.5×10^{-5}	Kp and K_M	3.75	4	<2% >1%
2.5×10^{-5}	V_{MAX} and Vp	18.09	4	<0.1
5.0×10^{-5}	Kp and K_M	15.63	1	<5% >2.5%
5.0×10^{-5}	V_{MAX} and Vp	107.62	1	<1% >0.5%
10^{-4}	Kp and K_M	36.84	1	<2% >1%
10^{-4}	V_{MAX} and Vp	22.40	1	<5% >2.5%

and to the mean Vp values obtained at these concentrations. These results are given in Table 58. It was found that raising [I] from 50 to 100 μ mol/l produced a significant rise in Kp, the effective Michaelis constant. The associated shift in the double reciprocal plot resulted in there being no significant difference between Vp at the concentration [I] of 100 μ mol/l and at each of the other two concentrations [I] . The point of intersection of the line when [I] was 100 μmol/l with the line when [I] was 0 moved nearer to the ordinate (Fig 15).

As the results under discussion suggested that the inhibition produced by TBBI was mixed values for K_i were not derived from these plots. This was because mixed inhibition implies a mixture of partially competitive inhibition with some form of non-competitive inhibition and K_i values cannot be accurately derived for partially competitive inhibitors by simple graphical means (Dixon and Webb, 1964).

(b) Experiments with other benzotriazinium compounds.

In order to ascertain whether the other three series of benzotriazinium compounds were likely to have a significantly different mode of anti-AcChE action from TBBI preliminary experiments were done with PMBI, BBBI and A5BI representing the 4-phenethylamino-, 4-benzylamino- and 4-anilino-series respectively.

Four experiments were performed with PMBI and A5BI and three with BBBI. Each experiment involved the study of the reaction rates at the four concentrations [S] (0.25, 0.5, 1.0 and 2.0 m mol/l) both in the absence and presence of a single concentration [I] . The concentration [I] chosen for each compound was 50 μmol/l for PMBI and A5BI and 100 μmol/l for BBBI.

The parameters K_M, Kp, V_{MAX} and Vp were calculated for each compound by double reciprocal plots as described for TBBI. Mean values for these parameters are shown in Table 59.

Table 58

Results of 't' tests between mean kinetic parameters Kp at different concentrations [I] and between mean kinetic parameters Vp at different concentrations [I]

Test between	't' (calc)	df	t(theo) P=0.95
Kp ([I] 100μ mol/l) and Kp ([I] 50μ mol/l)	17.72*	2	4.30
Kp ([I] 100μ mol/l) and Kp ([I] 25μ mol/l)	7.73*	6	2.45
Kp ([I] 25μ mol/l) and Kp ([I] 50μ mol/l)	0.26	6	2.45
Vp ([I] 100μ mol/l) and Vp ([I] 50μ mol/l)	4.25	2	4.30
Vp ([I] 100μ mol/l) and Vp ([I] 25μ mol/l)	2.31	6	2.45
Vp ([I] 25μ mol/l) and Vp ([I] 50μ mol/l)	4.22*	6	2.45

* Differences significant (P=0.95).

Table 59

Mean kinetic parameters determined for the AcCh-AcChE reaction in the presence and absence of three benzotriazinium compounds at pH 7.0 and 37°C.

Benzotriazinium compound.	Mean K_M value (m mol/l)	Mean Kp value (m mol/l)	Mean V_{MAX} value (μ moles AcCh hydrolysed per min).	Mean Vp value (μ moles AcCh hydrolysed per min.)
PMBI	0.2715	0.3958	1.3111	0.8368
	(0.0227)	(0.0415)	(0.0324)	(0.0272)
BBBI	0.2683	0.4788	1.5199	0.8111
	(0.0227)	(0.0771)	(0.0333)	(0.0349)
A5BI	0.2980	0.4023	1.5277	0.9772
	(0.0142)	(0.0275)	(0.0215)	(0.0179)

Figures in brackets are standard errors.

It was noted that for each experiment performed with each compound the value of Kp was always greater than the value for K_M. Similarly the value for V_{MAX} was always greater than the corresponding value Vp. The significance of these differences was tested by the calculation of values for 't' which are given in Table 60 . The results of the 't' tests showed that with the use of both PMBI and A5BI under the given conditions significant differences existed both between K_M and Kp values and between V_{MAX} and Vp values. This showed the production of mixed inhibition by these compounds. They thus resembled TBBI when tested in a comparable manner.

By contrast no significant difference between the K_M and Kp values was detected for BBBI although a significant difference between V_{MAX} and Vp values was found. This suggested that, under the conditions of the test, BBBI was acting as a non-competitive inhibitor of AcChE. It may have been, however, that the lack of significant difference in the K_M and Kp values reflected the use of only two degrees of freedom in the comparison and the fact that as earlier results from the AcCh-AcChE reaction had shown, Michaelis constants were subject to more variation that the V_{MAX} values.

Table 60

Values for 't' calculated from the differences between Kp and K_M and V_{MAX} and Vp from reactions between AcCh and AcChE at pH 7.0 and 37°C. in the presence and absence of TBBI

Compound	test between differences in	calculated value of 't'	df	Probability that differences are due to chance.
PMBI	Kp and K_M	4.16	3	<5% >2.5%
PMBI	V_{MAX} and Vp	10.76	3	<0.5% >0.1%
BBBI	Kp and K_M	3.09	2	> 5%
BBBI	V_{MAX} and Vp	31.69	2	< 0.1%
A5BI	Kp and K_M	4.57	3	<2% >1%
A5BI	V_{MAX} and Vp	22.31	3	<0.1%

Consistency of results with TBBI as AcChE inhibitor.

The present research permitted an assessment of the consistency with which the AcChE inhibition produced by TBBI could be measured over a period of time under narrowly defined conditions.

From experiments at pH 7.0 and 37°C and with concentrations of enzyme (0.05 units/ml), substrate (1.0 m mol/l) and TBBI (25 μ mol/l) the following percentage inhibitions were measured: 44.92% on the first occasion, percentage inhibitions within the range 36.07% to 45.90% after three months and an inhibition of 40.53% after ten months.

When the concentration of TBBI was 50 μ mol/l percentage inhibitions of 58.69% on the first occasion, 51.61% after ten months and 59.35% after fourteen months were measured.

At a concentration of TBBI of 100 μ mol/l percentage inhibitions ranging between 60.28% and 65.12% were measured over a period of five months.

These results suggested that the pH - stat technique, under the chosen conditions, could measure TBBI-induced inhibition of AcChE with a reasonable precision over a period of between five and ten months.

The effect of pH on the inhibition of AcChE produced by TBBI at 37°C

In view of the fact that insoluble and biologically inactive zwitterions might be formed at pH values of 7.4 and above for the 4-p-tolylamino-series of benzotriazinium compounds (Cull and Scott, 1973), a pilot experiment of the effect of pH on the action of TBBI was performed.

The experiment involved the determination of reaction velocities at 37°C for the AcCh-AcChE reaction in the presence and absence of TBBI (50 μ mol/l) at each of six pH values between pH 7.0 and pH 8.0. The pH values were 7.0, 7.2, 7.4, 7.6, 7.8 and 8.0. From the reaction velocities the percentage inhibitions of AcChE at these pH values were determined and were:-

pH	7.0	7.2	7.4	7.6	7.8	8.0
% inhibitions of AcChE	51.61	55.16	47.27	50.35	48.94	51.04

This result showed an apparently random variation in the inhibition of AcChE produced by TBBI, within the overall range of 47% to 55% inhibition, over the pH range tested. It was concluded that the effect of TBBI on erythrocyte AcChE at 37°C was not likely to be significantly modified by pH changes in the range pH 7.0 to pH 8.0.

The effect of TBBI on the reaction between AcCh and AcChE from electric eel at pH 7.0 and 37°C.

In the Introduction to this thesis it was pointed out that different forms of AcChE may show differences in structure. It was decided, therefore, to perform pilot experiments in which the effect of TBBI was examined on the reaction between AcCh and AcChE from a source other than bovine erythrocytes. For this purpose AcChE from electric eel was selected and used at the same concentration of 0.05 units/ml as the erythrocyte enzyme in previous experiments.

The reaction between AcCh (0.25 to 2.0 m mol/l) and this enzyme was characterised by the determination of a K_M value from each of two experiments. This mean K_M value was 0.1884 m mol/l(standard error 0.0568).

Three experiments were performed to determine I_{50} values for TBBI. Each experiment involved the reaction between a 1.0 m mol/l concentration AcCh and each of three concentrations TBBI within the range 6.25 to 50 μ mol/l. From a comparison between the reaction rate for the AcCh with the enzyme alone (v) and in the presence of TBBI (v^1) the ratios $\frac{v}{v_1}$ were derived. These were plotted against [TBBI] and regression analyses applied to these plots. The errors of these regression lines $\left(\frac{Sb}{b}\right)$ x 100% were within the range 2 to 6% and pointed to the linearity of this relationship. The I_{50} values were determined from substitution of a value of $\frac{v}{v_1}$ equal to 2 in the regression equations.

The mean I_{50} value for TBBI was 14.5 μ mol/l (standard error 1.0 μ mol/l) and compared with the I_{50} value of 34 μ mol/l recorded for TBBI from the reaction between AcChE (1.0 m mol/l) and erythrocyte enzyme (Table 49).

This result suggested that the potency of TBBI as an inhibitor of AcChE might differ with the type of AcChE under test.

General Discussion

The present research was undertaken to examine a series of benzotriazinium compounds for anti-acetylcholinesterase activity. The intention was to measure any such activity; to attempt to elucidate the mechanism of this activity where present; and to assess the likelihood that such an action might have influenced the pharmacological actions of these compounds on skeletal muscle preparations reported by Cull (1972).

In the event the major part of the present research has been devoted to an examination and comparison of the Warburg manometric method and the pH - stat method for the measurement of AcCh-AcChE reactions. These methods were selected from a wide variety of possible methods on the basis of the availability of the appropriate apparatus and the claims made for their accuracy and precision.

The Introduction to this thesis showed how many factors could affect the measured activity of a cholinesterase enzyme. The type, source and purity of the cholinesterase; its reaction with a given substrate and their relative concentrations; the effect of pH, inorganic ions, the presence of buffers and the temperature of the reaction were amongst such factors mentioned. It was decided, therefore, to concentrate most of the research on one form of AcChE studied under closely defined experimental conditions.

The Discussion on results from the Warburg manometric experiments pointed out that claims for the precision and accuracy of a particular experimental method may be somewhat equivocal particularly with reference to routine determinations. Thus it was considered important to investigate the chosen methods in respect of their accuracy and precision under routine conditions. So far as accuracy was concerned the only criteria for comparison were generally accepted values in the literature for given parameters derived under

similar experimental conditions. For example, values for K_M within the range 0.1 to 1.0 m mol/l are generally to be expected for the AcCh-AcChE reaction (Barlow, 1964). It became clear that there was a need to investigate precision at more than one stage in the derivation of the results from experiments. For example the precision with which reaction velocities obtained with given concentrations of enzyme and of substrate were derived was assessed in respect of the experimental technique used and the treatment of recorded data. Additionally similar assessments were made in respect of the derivation of the related kinetic parameters K_M and V_{MAX}. This type of information from cholinesterase determinations is not generally available in the literature and it was felt that such studies must precede any attempt to apply the methods for AcChE determination to the investigation of the benzotriazinium compounds. Inter-laboratory variations in results and the need for quality control procedures in the measurement of cholinesterase activities have been emphasized by Serat and Mengle (1973).

Of particular interest was the finding from the pH - stat experiments that the precision of the kinetic parameters K_M and V_{MAX} derived from the double reciprocal plot of $\frac{1}{v}$ on $\frac{1}{[S]}$ were not significantly worse than those derived by a plot of v on $\frac{v}{[S]}$. The values of these parameters were of the same magnitude when derived by the two plots. The condemnation of the use of the double reciprocal plot for the determination of kinetic parameters (Colquhoun, 1969, 1971) has not been borne out under the experimental conditions of the present research. It was noted that Dixon and Webb (1964) expressed a preference for the double reciprocal plot.

The present research included an investigation of certain factors, such as the influence of the substrate and enzyme preparations and the volumes of added reagent, which might have influenced the accuracy of the results.

It was concluded that of the two chosen experimental methods the pH - stat technique has afforded the greater precision and convenience.

Table 17 for example showed that the overall coefficient of variation in twenty-four measurements by the Warburg manometric method of the velocity of reaction between AcCh (2.0 m mol/l) and AcChE (0.05 units/ml) at 37°C was 13.52%. These results were obtained with the use of a single master enzyme solution (M.E.S). When the reaction velocities calculated from fifty-one reactions between these concentrations of enzyme and substrate but using the pH - stat method were examined, an overall coefficient of variation of 10.06% was found. However, this variation covered reaction velocities obtained from the use of eight different M.E.S. Coefficients of variation in the velocities obtained with the eight individual M.E.S varied from 2.00% to 8.39% (Table 36), five of the eight coefficients being less than 5%.

Whilst the pH-stat method only permitted the measurement of one reaction velocity at a time this velocity was measured over a shorter period of time and with few manipulations than a corresponding measurement of reaction velocity by Warburg manometery. Wilhelm, Vandekar and Reiner (1973) supported the use of quick methods of determining cholinesterase inhibition where there was a possibility of spontaneous reactivation of enzyme from an unstable complex with inhibitor. Equally they stated that an assay should not be so long as to permit further enzyme inhibition during its course. The graphical representation of a reaction on the pH - stat titrigraph permitted a rapid evaluation of that part of the curve which was to be used to calculate reaction velocity. From this a tangent could be quickly fitted to the curve at the appropriate point or the digital read-out of titrant added over that period easily recalled for the measurement of the velocity. By contrast, using the Warburg method, each reaction

velocity calculation involved the fitting of a regression line to a series of points, each point being, in turn, computed from four readings.

Thus the pH - stat method was chosen for an examination of the benzotriazinium compounds and physostigmine as anti-acetylcholinesterase compounds.

It should be borne in mind that the chosen method of assay may have influenced the measurement of the inhibitor activity by virtue of the restraints imposed by the chosen experimental variables. In this context it is important to realise that the reactivity of a membrane-bound cholinesterase 'in situ' towards inhibitors and substrates may be different from its reactions in solution.(Ehrenpreis et al 1969).

Experiments with the pH - stat method confirmed the anti-AcChE activity of PMBI and BnPBI previously tested by Warburg manometry. Additionally the pH-stat method has shown that anti-AcChE activity is a property shown by all the members of the four series of benzotriazinium compounds tested for pharmacological activity by Cull (1972).

The relative weakness of the benzotriazinium compounds tested as AcChE inhibitors, as measured by I_{50} values has already been remarked. This point may be confirmed by an examination of data concerning compounds tested as anti-cholinesterases. As examples, the p I_{50} values listed by Barlow (1964) and Usdin (1970) and the I_{50} values listed by Metcalf (1971) for many test compounds of differing structures may be consulted.

Usdin (1970) pointed out, however, that p I_{50} values are meaningless unless given in the context of the experimental conditions in which they were derived. He quoted p I_{50} values for physostigmine ranging from 7.0 to 8.0 and derived by the use of various combinations of pH, temperature and inhibitor contact time.

He also emphasized that inhibition is a function not only of the inhibitor but also of the enzyme. Here p I_{50} values for D.F.P were quoted which varied for AcChE from two different sources and for ψ ChE from two different sources. Thus comparisons of I_{50} values with results in the literature may often be less than precise.

It should also be emphasized that the meaning of a p I_{50} value for a reversible inhibitor in relation to the kinetic parameter K_i will only be clear if the type of inhibition produced and the characteristics of the enzyme studied are known (Barlow,1964). For TBBI the reversibility of its inhibition of AcChE was established.

It was shown in the case of physostigmine that kinetic behaviour may be altered by enzyme-inhibitor contact time. This adds further caution to the interpretation of the anti-AcChE activities of the benzotriazinium compounds except under the given experimental conditions.

It was noted that the interpretation of the results obtained with TBBI as AcChE inhibitor at pH 7.0 and 37°C and after an incubation time of three minutes could be influenced by their mathematical treatment. The plots of Dixon (1953) and of Hunter and Downs (1945) applied to results obtained with TBBI as AcChE inhibitor and examined in isolation permitted the conclusion that the inhibition produced was competitive. This would allow estimates of K_i to be made, as was done in this research. The estimates were that the value of K_i lay within the range 27.2 to 40.4 μ mol/l depending upon the method of derivation. Double-reciprocal plots, however, suggested that inhibition was mixed, a situation not permitting calculation of accurate K_i values from the data presented in the present research. This type of inhibition was also found for PMBI and A5BI under the same conditions. It suggested that as well as complexes between enzyme and substrate and between enzyme and inhibitor, complexes of enzyme with both substrate nnd inhibitor may be formed (Dixon and Webb,1964) .

Again caution in the interpretation of literature data may be required. Robinson and Robinson (1968) calculated a K_i value for physostigmine when inhibition was shown to be mixed from the slope of the double reciprocal plot in the presence of the inhibitor. They apparently used the expression:-

$$\text{gradient} = \frac{K_M \left(1 + \frac{[I]}{K_i}\right)}{V_{MAX}}$$

This relationship holds only for fully competitive or fully non-competitive inhibitors (see Zeffren and Hall, 1973).

From experiments performed with physostigmine it was shown that the precision of K_i values may be influenced by the graphical parameter used in its derivation. The K_i values derived in situations where the inhibition was established as non-competitive were shown to have variances which were significantly greater when derived from the slope of the double reciprocal plot rather than from the intercept of the plot with the ordinate.

That the benzotriazinium compounds have anti-AcChE activity may, perhaps, have been anticipated from the literature. Baker, Ho and Santi (1965) and Baker and Ho (1966) tested compounds with the s-triazine structure as inhibitors of the enzyme dihydrofolic reductase. Recently Sacher, Alt and Darlington (1973) reported on the effectiveness of a series of benzotriazolyl methyl and dimethyl-carbamates as inhibitors of fly and bovine erythrocyte cholinesterase. The I_{50} values for these compounds ranged from 0.4 μmol/l to 80 μ mol/l against the fly enzyme and from 8 μmol/l to 500 μmol/l against the bovine enzyme. The latter values are comparable with the values found in the present research against this enzyme. In his Table of insecticides thought to inhibit AcChE Corbett (1974) included compounds in which triazine structures were linked to phosphorus containing groups.

The explanation of the biological action of the benzotriazinium compounds in molecular terms is not yet clear. Baker, Ho and Santi (1965) and Baker and Ho (1966) stressed the importance of hydrophobic bonding between substituted s-triazines and dihydrofolic reductase. Kabachnik, Brestkin, Godovikov, Michelson, Rozengart and Rozengart (1970) reviewed the evidence for the existence and disposition of hydrophobic areas on the active surface of both butyrylcholinesterase and acetylcholinesterase. They suggested that these areas may serve to throw a hydrophilic substrate molecule such as acetycholine into contact with the active centre of the enzyme and so facilitate their reaction. Further it was suggested that the binding between hydrophobic areas away from the active site of the enzyme and bulky hydrophobic groups on drug molecules led to blockade of the receptor area by hindering desorption of the drug from the receptor. It is possible that such a mechanism may obtain with the benzotriazinium compounds.

Cull (1972) reported for each of the four series of benzotriazinium compounds, the clear increase of agonist activity on both the frog rectus abdominis and chick biventer cervicis preparations with increase in the length of the n-alkyl side chain attached to the quaternary nitrogen atom. This reached a maximum with the n-butyl analogue. She also showed that whilst lipid solubility was of importance in agonist activity the relationship between these variables was not consistent for all compounds. Evidence was presented that in the frog rectus abdominis preparation alterations to the alkyl side chain were having a greater effect on activity than changes in the 4-aryl groups. With the chick biventer cervicis preparation the effects of altering the two groups was not markedly different. This led to the suggestion that the benzotriazinium binding sites in the different preparations might differ structurally.

The fact that the benzotriazinium compounds showed no immediately

apparent relationship between structure and anti-AcChE activity is perhaps not surprising. Cull and Scott (1973) proposed that the compounds were acting on the chick and frog tissues like quinine and quinidine. They pointed out that if the compounds were acting through an acetylcholine receptor an increase of agonist activity with increase in the size of the alkyl group attached to the quaternary nitrogen atom would not be expected. Antagonism of the agonist effects by tubocurarine would also be expected, but this was not seen. The resemblance of the neuromuscular effects of the benzotriazinium compounds to those of quinidine was also reported by Khan (1973). In addition he showed that the benzotriazinium compounds possessed cardiac depressant activity. Thus their pharmacology is complex as possibly also is their activity at a molecular level.

It is clear that in respect of their anti-AcChE activities further experiments should be performed with the benzotriazinium compounds. These should include both deeper kinetic studies and a further investigation of structure-activity relationships in order to shed more light on the mechanism of their cholinesterase inhibition. Such studies should encompass a wider range of variables than presently tested and may be conveniently performed by the use of the pH - stat technique.

Of particular relevance here was the finding, from a pilot experiment, that TBBI was approximately twice as potent against eel AcChE as against the bovine erythrocyte enzyme. This confirmed the finding of Sacher et al (1973) that triazine-containing inhibitors may have marked differences in potency against different types of cholinesterase. It would be informative, in view of the neuromuscular activities of the benzotriazinium compounds, to test them against cholinesterase from such a tissue. This might not be straightforward. Berry and Rutland (1971) for example produced evidence that diaphragm muscle of guinea pig, man and rat contained

both soluble and particulate AcChE (E.C,3.1.1.7) and a soluble cholinesterase (E.C.3.1.1.8).

In general it may be concluded that with the emphasis in the present research being placed on the assessment of AcChE activity, the possible contribution that anti-AcChE activity has made to the pharmacological actions of the benzotriazinium compounds on skeletal muscle preparations has not been elucidated as hoped. The doses of benzotriazinium compounds producing 50% inhibition of AcChE are of the same magnitude as those reported by Cull (1972) and Khan (1973) as producing pharmacological effects on skeletal muscle. It must be borne in mind, however, that in the present research the benzotriazinium compounds have been applied directly to the enzyme preparation. In the case of the pharmacological preparations tissue barriers to the access of thsse compounds might mean that the concentrations able to reach the AcChE in such preparations are lower than those used in the present research. This possibility lends support to the need for a future study of the benzotriazinium compounds as inhibitors of neuromuscular AcChE, preferably in parallel with similar studies on quinine and quinidine whose pharmacological properties are said to be similar to those of the benzotriazinium compounds.

BIBLIOGRAPHY.

Aldrich,F.D., Walker,G.F.,&Patnoe,C.A.(1969). A micromodification of the pH-stat assay for human blood cholinesterase. Arch.environ. Health, 19, 617-620

Alles,G.A. & Hawes, R.C. (1940). Cholinesterses in the blood of man. J. Biol.Chem., 133, 375-390.

Ammon, R. (1933). Die fermentative spaltung des acetylcholins. Pflugers Arch. ges. Physiol., 233, 486-491

Anderson, A.E. (1973). Disubstituted -1,2,3-benzotriazines as inhibitors of acetylcholinesterase. BSc Dissertation, Heriot-Watt University, Edinburgh.

Anon. (1961). Report of the Commission on Enzymes of the International Union of Biochemistry (1961). Oxford : Pergamon.

Anon. (1965). Enzyme Nomenclature. Recommendations (1964) of the International Union of Biochemistry on the Nomenclature and Classification of Enzymes together with their Units and the Symbols of Enzyme Kinetics. Amsterdam : Elsevier.

Ashani,Y & Wilson, I.B. (1972). A covalent affinity column for the purification of acetylcholinesterase. Biochim.biophys. Acta, 276 , 317-322.

Augustinsson, K-B. (1948). Cholinesterases. A study in comparative enzymology. Acta physiol. scand., 15, Supplement 52.

Augustinsson, K-B. (1949). Substrate concentration and specifidty of choline ester-splitting enzymes. Archs Biochem., 23, 111-126.

Augustinsson, K-B. (1955a). A titrimetric method for the determination of plasma and red blood cell cholinesterase activity using thiocholine esters as substrates. Scand.J. clin. Lab. Invest., 7 , 284-290.

Augustinsson, K-B. (1955b). The normal variation of human blood cholinesterase activity. Acta physiol. scand., 35, 40-52.

Augustinsson, K-B. (1957). Assay methods for cholinesterases. Meth. biochem. Analysis, 5, 1-63.

Augustinsson, K-B. (1963). Classification and comparative enzymology of the cholinesterases and methods for their determination. In 'Cholinesterases and anti-cholinesterase agents', Handb. exp. Pharmac. Vol.15, ed. Koelle, G.B. pp89-128. Berlin: Springer-Verlag.

Augustinsson, K-B. (1971). Determination of activity of cholinesterases. Meth. biochem. Analysis, Supplement, 217-273.

Augustinsson, K-B. & Holmstedt, B. (1965). Determination of cholinesterase in blood samples dried on filter paper and its practical application. Scand.J. clin. Lab. Invest., 17, 573-83.

Baker, B.R. & Ho, B.T. (1966). Analogs of tetrahydrofolic acid XXXV. Hydrophobic bonding to dihydrofolic reductase. V11 Species differences with substituted 4,6-diamino - 1,2-dihydro-s-triazines. J. pharm. Sci., 55, 470-474

Baker, B.R., Ho, B.T. & Santi, D.V. (1965). Analogs of tetrahydrofolic acid XXVII Hydrophobic bonding to dihydrofolic reductase by 2-amino-4,6- disubstituted-5-alkyl-pyrimidines and 1-alkyl - 4,6 - diamino - 1,2 - dihydro -s-triazines. J. pharm. Sci., 54, 1415-1424.

Ballantyne, B. (1968a). Histochemical and biochemical aspects of cholinesterase activity of adipose tissue. Archs int. Pharmacodyn. Ther, 173, 343-50

Ballantyne, B. (1968b). Potentiometric pH-stat titration. Importance of an inert atmosphere in reaction vessels when using alkali titrant. Experientia, 24, 329-330.

Barlow, R.B. (1964). Introduction to chemical pharmacology. 2nd Ed. London: Methuen & Co. Ltd.

Baron, R.L., Casterline, J.L., Jr. & Orzel, R. (1966). In vivo effects of carbamate insecticides on mammalian esterase enzymes. Toxic. appl. Pharmac., 9, 6-16.

Baum,G. (1971). Determination of acetylcholinesterase by an organic substrate selective electrode. Anal Biochem., 39, 65-72.

Baum, G., Ward, F.B. & Yauerbaum, S. (1972). Kinetic analysis of cholinesterases using a choline ester selective electrode. Clinica chim. Acta, 36, 405-408.

Beckett, A.H., Vaughan, C.L. & Mitchard, M. (1969). Stability studies on crude and purified horse serum cholinesterase. Biochem. Pharmac., 18, 1701-1705.

Berman, J.D. (1973). Structural properties of acetylcholinesterase from eel electric tissue and bovine erythrocyte membranes. Biochemistry, 12, 1710-1715.

Berman, J.D. & Young, M. (1971). Rapid and complete purification of acetylcholinesterases of electric eel and erythrocyte by affinity chromatography. Proc. natn. Acad. Sci. U.S.A., 68, 395-398.

Berry, W.K. & Rutland, J.P. (1971). Choline ester hydrolases in diaphragm muscle. Biochem. Pharmac., 20, 669-682

Bhatnager, S.P. (1968). The relationship between red cell cholinesterase levels and erythropoiesis induced by haemorrhage and cobalt treatment in rabbits. Arch int. Pharmacodyn. Ther, 175, 422-439.

Bockendahl, H.& Ammon, R. (1965). Cholinesterases. In 'Methods in enzymatic analysis', ed. Bergmeyer H.-V. pp 771-775. New York: Academic Press.

Boutin, D. & Brodeur, J. (1970). An automated method for simultaneous determination of serum pseudocholinesterase activity, dibucaine number and fluoride number. Clin. Biochem., 3, 245-254.

Brestkin, A.P., Brik, I.L., Volkova, R.I., Maizel, E.B. & Rozengart, E.V. (1970). Influence of ionic strength and organic solvents on interaction of cholinesterases with substrates and organophorphorus inhibitors. Biokhimiya, 35, 317-326.

Brestkin, A.P & Ivanova, L.A. (1970). The effect of lithium and calcium ions on the activity of erythrocyte - derived acetylcholinesterase. Biokhimiya, 35, 575-578.

Briggs, G.E. & Haldane, J.B.S. (1925). A note on the kinetics of enzyme action. Biochem. J., 19, 338-339.

Brown, H.V. & Bush, A.F. (1950). Parathion inhibition of cholinesterase. Archs. ind. Hyg., 1, 633-636.

Buckley, G.A. & Heaton, J. (1970). Simultaneous determination of choline acetylase and cholinesterase activity. Br.J.Pharmacol., 40, 591P-592P.

Buckley, G.A. & Nowell, P.T. (1966). Micro-colorimetric determination of cholinesterase activity of motor end plates in the rat diaphragm. J. Pharm. Pharmac., 18, 146S-150S.

Bulletin of The World Health Organisation (1971). Alternative Insecticides for Vector Control. Bull. Wld.Hlth Org., 44, 1-470.

Carr, C.J. & Bell, F.K. (1947). The pharmacology of a new series of choline salts. J.Pharmac. exp. Ther., 91, 169-173.

Cavallito, C.J. (1968). Some relationships between chemical structure and pharmacological activities. A. Rev. Pharmacol., 8, 39-66.

Chang, H.C. & Gaddum, J.H. (1933). Choline esters in tissue extracts. J.Physiol., London., 79, 255-285.

Changeux, J.-P. (1966). Responses of acetylcholinesterase from Torpedo mamorata to salts and curarising drugs. Mol.Pharmacol., 2, 369-392.

Chiu, Y,C., Tripathi, R.K. & O'Brien, R.D. (1972). A gel-scanning method for kinetic studies on an acetylcholinesterase isozyme. Anal Biochem., 45, 480-487.

Chouteau, J., Rancien, P.& Karamanian, A. (1956). Recherches sur les estérases du serum sanguin. Bull. Soc. Chim. biol., 38, 1329-1336

Clitherow, J.W., Mitchard, M. & Harper, N.J. (1963). The possible biological function of pseudocholinesterase. Nature. 199, 1000-1001.

Cohen, J.A. & Oosterbaan, R.A. (1963). The active site of acetylcholinesterase and related esterases and its reactivity towards substrates and inhibitors. In 'Cholinesterases and anticholinesterase agents', Handb. exp. Pharmac. Vol 15, ed. Koelle, G.B. pp 299-373. Berlin: Springer-Verlag.

Colquhoun, D. (1969). A comparison of estimators for a two-parameter hyperbola. Jl R. statist. Soc. Ser C., 18, 130-140.

Colquhoun, D. (1971). Lectures on biostatistics. London:Oxford University Press.

Coppage, D.L. (1971). Characterization of fish brain acetylcholinesterase with an automated pH stat for inhibition studies. Bull. Environ. Contam. Toxicol., 6, 304-310.

Corbett, J.R. (1974). Insecticides inhibiting acetylcholinesterase. In 'The biochemical mode of action of pesticides'. pp 107-164. London & New York: Academic Press.

Crammer, M.F. & Peoples, A. (1971). A sensitive gas chromatographic method for human cholinesterase determination. J. Chromat., 57, 365-371.

Crone, H.D. (1973). The influence of ionic strength on the interaction of quaternary drugs with mammalian acetylcholinesterase in relation to possible regulatory effects. J. Neurochem., 20, 225-236.

Cull, G.A.G. (1972). The pharmacology of some 1,2,3-benzotriazines. Ph.D. Thesis: Heriot-Watt University, Edinburgh.

Cull, G.A.G. & Scott, N.C. (1973). Structure-activity relationships in some series of 2-alkyl-1,2,3-benzotriazinium compounds. Br.J. Pharmacol., 47, 819-826.

Dale, F.J. & Robinson, B. (1970). The synthesis and anti-acetylcholinesterase activities of (+)-physostigmine and (+) - physovenine. J. Pharm. Pharmac., 22, 889-896.

Delaunois, A.L. (1962). Automatized micromethod for the potentiometric determination of cholinesterase activity. Archs int. Pharmacodyn. Ther., 140, 351-357.

Delaunois, A.L. & Casier, H. (1946). Microméthode potentiometrique pour la détermination de la cholinesterase. Experientia, 2, 67-69.

Delaunois, A.L. & Casier, H. (1948). Micro-méthode potentiometrique pour la détermination de l'activité des cholinesterases. Archs int. Pharmacodyn. Ther, 75, 371-381.

Dixon, M. (1952). Manometric methods as applied to the measurement of cell respiration and other processes. 3rd Ed Cambridge:Cambridge University Press.

Dixon, M. (1953). The determination of enzyme inhibitor constants. Biochem. J., 55, 170-171.

Dixon, M.& Webb, E.C. (1964). Enzymes. 2nd Ed. London: Longmans.

Dowd, J.E.& Riggs, D.S. (1965). A comparison of estimates of Michaelis-Menten kinetic constants from various linear transformations. J.Biol. Chem., 240, 863-869.

Dubois, K.P., Kinoshita, F.K. & Frawley, J.P. (1968). Quantitative measurement of inhibition of aliesterases, acylamidase and cholinesterase by EPN and Delnav. Toxic. appl. Pharmac., 12, 273-284

Ehrenpreis, S., Fleisch, J.H. & Mittag, T.W. (1969). Approaches to the molecular nature of pharmacological receptors. Pharmac. Rev., 21, 131-181.

Ehrenpreis, S., Mittag, T.W. & Patrick, P. (1970). Radiometric assay of cholinesterases in intact tissues in the nanomolar concentration range of acetylcholine. Biochem. Pharmac., 19, 2165-2169.

Ellin, R.I. (1972). Cholinesterase determination. Clin. Chem., 18, 487.

Ellin, R.I., Groff, W.A. & Kaminskis, A. (1972). An error-producing interaction in an automated method for measuring cholinesterase activity in blood. Clin.Chem., 18, 1009-1012.

Ellman, G.L., Courtney, K.D., Andres, V. Jr. & Featherstone, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmac., 7, 88-95.

Engelhard, N., Prchal,K. & Nenner, M. (1967). Acetylcholinesterase. Angew. Chem., Int. Ed. Engl., 6, 615-626.

Eränko,O. (1967). Histochemistry of nervous tissues: Catecholamines and cholinesterases. A. Rev. Pharmacol., 7, 203-222.

Fried, R. & Howse,M. (1971). A demonstration of enzyme activity for the 'Sceptical Chymist'. J. chem. Educ., 48, 847-848.

Geigy. (1970). Documenta Geigy. Scientific Tables, 7th Ed., ed. Diem, K. & Lentner, C. Basle: J.R. Geigy. S.A.

Gillette, J.R. (1971). Techniques for studying drug metabolism in vitro. In 'Fundamentals of drug metabolism and drug disposition'. ed., La Du, B.N., Mandel, H.G.& Way, E.L. pp 400-416. Baltimore: Williams & Wilkins.

Glick,D. (1937). Properties of cholinesterase in human serum. Biochem. J., 31, 521-525.

Goldstein, A. (1951). Properties and behavior of purified human plasma cholinesterase. III Competitive inhibition by prostigmine and other alkaloids with special reference to differences in kinetic behavior. Archs Biochem., 34, 169-188.

Goodson, L.H., Jacobs, W.B. & Davis, A.W. (1973). An immobilized cholinesterase product for use in the rapid detection of enzyme inhibitors in air or water. Anal Biochem., 51, 362-367.

Goyer, G.R. (1968). Évaluation par méthode manométrique des cholinestérases des muscles diaphragme et tibial antérieur chez le rat. Revue can. Biol., 27, 209-216.

Hall, G.E. & Lucas, C.C. (1937). Choline-esterase activity of normal and pathalogical human serum. J. Pharmac. exp. Ther., 59, 34-42.

Hanes, C.S. (1932). Studies on plant amylases. 1. Effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. Biochem.J., 26, 1406-1421.

Hardegg,W. & Schaefer,H. (1952). Zur kinetik der cholinesterasen. Pflügers Arch. ges. Physiol., 255, 136-153.

Heffron, P.F. (1972). Actions of the selective inhibitor of cholinesterase, tetramono-isopropyl pyrophosphortetramide on the rat phrenic nerve-diaphragm preparation. Br.J. Pharmacol., 46, 714-724.

Hestrin, S. (1949). The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine, and its analytical applications. J.Biol.Chem., 180, 249-261.

Hobbiger, F. (1963). Reactivation of phosphorylated acetylcholinesterase. In 'Cholinesterases and anticholinesterase agents', Handb. exp. Pharmac. Vol 15, ed. Koelle, G.B. pp 921-988. Berlin: Springer-Verlag.

Hofstee, B.H.J. (1959). Non-inverted plots versus inverted plots in enzyme kinetics. Nature, 184, 1296-1298.

Holmstedt, B. (1963). Structure-activity relationships of the organophosphorus anticholinesterase agents. In 'Cholinesterases and anticholinesterase agents', Handb. exp. Pharmac. Vol. 15, ed Koelle, G.B. pp 428-485. Berlin:Springer-Verlag.

Hunter, A. & Downs, C.E. (1945). The inhibition of arginase by amino-acids. J. Biol.Chem, 157, 427-446.

Hunter, A.R. (1970). Determination of the serum cholinesterase. Br.J. Anaesth., 42, 872-874.

Ivanova, L.A. (1967). Effect of sodium ions on the activity of acetylcholinesterase of erythrocytes. Biokhimiya, 32, 975-979.

Jain, M.K., Mehl, L.E. & Cordes, E.H. (1973). Incorporation of eel electroplax acetylcholinesterase into black lipid membranes. A possible model for the cholinergic receptor. Biochem. biophys. Res. Commun., 51, 192-197.

Jensen-Holm, J., Lausen, H.H., Milthers, K. & Møller, K.O. (1959). Determination of cholinesterase activity in blood and organs by automatic titration. With some observations on serious errors of the method and remarks of the photometric determination. Acta pharmac. tox., 15, 384-394.

Jorgensen, K. (1959). Automatic recording of choline esterase activities. Scand.J. clin. Lab. Invest., 11, 282-284.

Kabachnik, M.I., Brestkin, A.P., Godovikov, N.N., Michelson, M.J., Rozengart, E.V. & Rozengart, V.I. (1970). Hydrophobic areas on the active surface of cholinesterases. Pharmac. Rev., 22, 355-388.

Kalow, W. & Lindsay, H.A. (1955). A comparison of optical and manometric methods for the assay of human serum cholinesterase. Can. J. Biochem. Physiol., 33, 568-574.

Karczmar, A.G. (1967a). Neuromuscular pharmacology. A. Rev. Pharmacol., 7, 241-276.

Karczmar, A.G. (1967b). Pharmacologic, toxicologic and therapeutic properties of anticholinesterase agents. In 'Physiological Pharmacology' Vol III - The Nervous System. Part C. ed. Root, W.S.& Hofmann, F.G. pp 164-322. New York & London: Academic Press.

Karczmar, A.G. (1970).(ed.) Anticholinesterase agents. Section 13. Vol.1. International Encyclopedia of pharmacology and therapeutics. Oxford: Pergamon.

Kato, G. (1968). Studies on serum cholinesterase kinetics by nuclear magnetic resonance spectroscopy. Mol. Pharmacol., 4, 640-644.

Kato, G. (1972a). Acetylcholinesterase. I. A study of nuclear magnetic resonance of the binding of inhibitors to the enzyme. Mol. Pharmacol., 8, 575-581.

Kato, G. (1972b). Acetylcholinesterase. II. A study of nuclear magnetic resonance of the acceleration of acetylcholinesterase by atropine and inhibition by eserine. Mol. Pharmacol., 8, 582-588.

Kato, G., Yung,J & Ihnat, M. (1970). Nuclear magnetic resonance studies on acetylcholinesterase. The use of atropine and eserine to probe binding sites. Mol. Pharmacol., 6, 588-596.

Kaufman, K. (1954). Serum cholinesterase activity in the normal individual and in people with liver disease. Ann. intern. Med., 41, 533-545.

Kay, J.B. (1968). Some derivatives of the aminocyclohexanols- their preparation and use in the study of some stereochemical factors in acetylcholinesterase catalysed hydrolysis and inhibition. Ph.D. Thesis; Manchester University.

Khan, M.T. (1973). A study of antiarrhythmic and neuromuscular blocking actions of 1,2,3-benzotriazine derivatives. Ph.D. Thesis: Heriot-Watt University, Edinburgh.

Koelle, G.B. (1963). Cytological distributions and physiological functions of cholinesterases. In 'Cholinesterases and anti-cholinesterase agents'. Handb. exp. Pharmac. Vol 15, ed. Koelle, G.B. pp 187-298. Berlin:Springer-Verlag.

Kramer, D.M., Cannon, P.L.Jr., Guilbault, G.G. (1962). Electrochemical determination of cholinesterase and thiocholine esters. Analyt.Chem., 34, 842-845.

Lineweaver, H.& Burk, D. (1934). The determination of enzyme dissociation constants. J. Am.chem.Soc., 56, 658-666.

Long, J.P. (1963). Structure-activity relationships of the reversible anticholinesterase agents. In 'Cholinesterases and anticholinesterase agents'. Handb. exp. Pharmac. Vol 15, ed. Koelle, G.B. pp 374-427. Berlin:Springer-Verlag.

Lyall, J. (1974). Unpublished work.

McOsker, D.E. & Daniel, L.J. (1959). A colorimetric micromethod for the determination of cholinesterase. Archs. Biochem. Biophys., 79, 1-7.

Mendel, B.& Hawkins, R.D. (1950). Estimation of the cholinesterases. Meth.med. Res,, 3, 107-115.

Metcalf, R.L. (1971). Structure-activity relationships for insecticidal carbamates. Bull. Wld.Hlth Org., 44, 43-78.

Michaelis, L. & Menten, M.L. (1913). Der kinetik der invertinwirkung. Biochem Z., 49, 333-369.

Michel, H.O. (1949). An electrometric method for the determination of red blood cell and plasma cholinesterase activity. J.Lab.clin. Med., 34, 1564-1568.

Mounter, L.A. (1963). Metabolism of organophosphorus anti-cholinesterase agents. In 'Cholinesterases and anticholinesterase agents'. Handb. exp. Pharmac. Vol 15, ed. Koelle, G.B. pp 486-504. Berlin:Springer-Verlag.

Myers, D.K. (1952). Effect of salt on the hydrolysis of acetylcholine by cholinesterases. Archs Biochem. Biophys., 37, 469-487.

Nabb, D.P. & Whitfield,F. (1967). Determination of cholinesterase by an automated pH stat method. Arch. environ. Health, 15, 147-154.

Nachmansohn, D. (1959). Chemical and molecular basis of nerve activity. New York & London: Academic Press.

Nachmansohn, D. (1963). Actions on axons, and evidence for the role of acetylcholine in axonal conduction. In 'Cholinesterases and anti-cholinesterase agents'. Handb. exp. Pharmac. Vol 15, ed Koelle, G.B. pp 701-740. Berline:Springer-Verlag.

Nachmansohn, D. (1966). Role of acetylcholine in neuromuscular transmission. Ann. N.Y. Acad, Sci., 135, 136-149.

Nachmansohn, D. & Scheenmann, H. (1945). On the effect of drugs on cholinesterase. J.Biol. Chem., 159, 239-240.

Nachmansohn,D. & Wilson, I.B. (1955). Acetylcholinesterase. Meth Enzym., 1, 642-651.

Neilands, J.B.& Cannon, M.D. (1955). Automatic recording pH instrumentation. Analyt. Chem., 27, 29-33.

Pavlic, M. (1967). The inhibitory effect of TRIS on the activity of cholinesterases. Biochim. biophys. Acta, 139, 133-137.

Radouco, C. & Frommel, E. (1952). Microméthode électrometrique pour la mesure de l'activité des cholinesterases. Helv. physiol. pharmac. Acta, 10, C39-C40.

Reed, D.J., Goto,K. & Wang, C.H. (1966). A direct radioisotopic assay for acetylcholinesterase. Anal Biochem., 16, 59-64.

Renshaw, R.R. & Bacon, N. (1926). The basis for the physiological activity of onium compounds. VI Rate of hydrolysis of certain esters of choline. J. Am. chem. Soc., 48, 1726-1732.

Robinson, B.& Robinson, J.B. (1968). The anti-acetylcholinesterase activities of the alkaloids of Physostigma venenosum seeds. J.Pharm. Pharmac., 20, 213S-217S.

Robinson, R. (1971). Clinical chemistry and automation. London: Charles Griffin & Co.Ltd.

Rosenberry, T.L., Chang, H.W. & Chen, Y.T. (1972). Purification of acetylcholinesterase by affinity chromatography and determination of active site stoichiometry. J. Biol. Chem., 247, 1555-1565.

Roufogalis, B.D. & Thomas, J. (1968a). Potentiatiation of acetylcholinesterase by a series of quaternary ammonium compounds. J. Pharm. Pharmac., 20, 135-145.

Roufogalis, B.D. & Thomas, J. (1968b). The acceleration of acetylcholinesterase activity at low ionic strength by organic and inorganic cations. Mol. Pharmacol., 4, 181-186.

Sacher, R.M., Alt, G.H. & Darlington, W.A. (1973). Insecticidal and anticholinesterase activity of benzotriazoyl methyl and dimethyl-carbamates. J. agric. Fd. Chem., 21, 132-135.

Saunders, L. & Fleming, R. (1966). Mathematics and statistics for use in pharmacy, biology and chemistry. Revised version. London: The Pharmaceutical Press.

Schatzberg-Porath, G., Zahavy, J & Gitter, S. (1963). Determination of cholinesterase inhibition with the use of a choline-dependent strain of Neurospora crassa. Nature, 198, 686-7.

Scoz,G. & Cattaneo, C. (1937). Determinazione del potere colino-esterasico del sangue per via elettrometrico. Enzmologia. 4, 157-162.

Serat, W.F.& Mengle, D.C. (1973). Quality control in the measurement of blood cholinesterase activities among persons after exposure to pesticides. Bull, Environ. Contam. & Toxicol., 9, 24-27.

Smallman, B.N. & Wolfe, L.S. (1954). The effect of salts on the estimation of cholinesterase activity. Enzymologia. 17, 133-144.

Stedman, E & Stedman, E. (1935). The relative choline-esterase activities of serum and corpuscles from the blood of certain species. Biochem. J., 29, 2107-2111.

Stedman,E., Stedman,E. & Easson, L.H. (1932). Choline-esterase. An enzyme present in the blood serum of the horse. Biochem. J., 26, 2056-2066.

Stedman,E., Stedman,E. & White, A.C. (1933). A comparison of the choline-esterase activities of the blood-sera from various species. Biochem. J., 27, 1055-1060.

Stein, H.H.& Lewis, G.J. (1969). Noncompetitive inhibition of acetylcholinesterase by eserine. Biochem. Pharmac., 18, 1679-1684,

Stevens, H.N.E. & Stevens, M.F.G. (1970a). Triazines and related products. Part III. Synthesis and re-arrangement of 3,4-dihydro-4-imino-1,2,3-benzotriazines. J. Chem. Soc. (C). 765-771.

Stevens, H.N.E. & Stevens, M.F.G. (1970b). Triazines and related products. Part V The structure of some red N-alkyl -1,2,3 - benzotriazines. J.Chem.Soc.(C). 2289-2298.

Uete,T., Tsuchikura, H. & Hoshida,K. (1970). A fluorimetric assay of the acetylcholinesterase activity in blood. Clin. Biochem. 3, 327-333.

Umbreit, W.W., Burris, R.H. & Stauffer, J.F. (1964). Manometric techniques. 4th Ed. Minneapolis:Burgess Publishing Co.

Usdin, E. (1970). Reactions of cholinesterases with substrates, inhibitors and reactivators. In 'Anticholinesterase agents', pp 47-354, Section 13, Vol 1, ed., Karczmar, A.G..of International Encyclopedia of pharmacology and therapeutics. Oxford: pergamon.

Waelsch, H & Nachmansohn,D. (1943). On the toxicity of Atabrine. Proc.Soc. exp. Biol Med., 54, 336-338.

Warburg, O (1926). Uber den stoffwechsel der tumoren. Berlin: Springer. English translation (1930). Dickens, F. London; Constable & Co.

Way, J.L.& Way, E.L. (1968). The metabolism of the alkyl phosphate antagonists and its pharmacological implications. A. Rev. Pharmacol., 8, 187-212.

Wilhelm, K., Vandekar, M. & Reiner, E. (1973). Comparison of methods for measuring cholinesterase inhibition by carbamates. Bull. Wld Hlth Org., 48, 41-44.

Wills, J.H.(1970). Toxicity of anticholinesterases and its treatment. In 'Anticholinesterase agents', pp 355-469, Section 13, Vol I, ed. Karczmar, A.G. of International Encyclopedia of pharmacology and therapeutics. Oxford: Pergamon.

Winteringham, F.P.W. & Disney, R.W. (1964). A radiometric study of cholinesterase and its inhibition. Biochem.J., 91, 506-514.

Witter, R.F. (1962). A critical study of the manometric assay of cholinesterase in rat blood. Toxic. appl. Pharmac., 4, 313-323.

Witter, R.F.(1963). Measurement of blood cholinesterase. A critical account of methods of estimating cholinesterase with reference to their usefulness and limitations under different conditions. Arch. environ. Health, 6, 537-563.

Wright, C.I. & Sabine, J.C. (1948). Cholinesterases of human erythrocyte and plasma and their inhibition by antimalarial drugs. J.Pharmac. exp. Ther., 93, 230-239.

Zeffren, E. & Hall, P.L. (1973). The study of enzyme mechanisms. pp 53-99. New York: John Wiley & Sons.

Zupancic, A.O. (1970). Kinetic response of a membrane-bound acetylcholinesterase to cholinergic activating and blocking agents. FEBS Lett. 11, 277-280.

Zupancic, A.O., Majcen, Z. & Stalc, A. (1972). Kinetics of muscarinic cholinoreceptors and of acetylcholinesterase of frog heart ventricle under the influence of atropine. Life Sci., 1. 11, 135-139.